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New Trends in β-Oxidation: Advances in the Study of α,β-Dehydrogenation

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Degradation of fatty acids as their CoA conjugates *via* β-oxidation has long been recognized as an important metabolic process. Studies on four enzymes involved, α,β-acyl-CoA dehydrogenase, hydratase (crotonase), β-OH-acyl-CoA dehydrogenase, and thiolase have progressed in an unsteady fashion, periods of activity being followed by such of relative dormancy.

In the past few years, however, it has been recognized that genetic defects concerning the enzymes involved in β-oxidation or those in fatty acid transport systems are relatively common, and might be related to the so-called "sudden infant death" syndrome. In this paper we will discuss the most recent progress in this field.

α,β-Dehydrogenation is a key process in β-oxidation, since it is most probably the rate-limiting step under physiological conditions. There are three different but related flavin-dependent enzymes catalyzing the α,β-dehydrogenation step. They are specific for long-chain acyl-CoA (LCAD), medium-chain acyl-CoA (MCAD), and short-chain acyl-CoA (SCAD). In addition, two similar enzymes that catalyze dehydrogenation of isovaleryl-CoA and of isobutyryl-CoA are known. Both enzymes play an important role in the degradation of the

CoA-conjugates that arise from the degradative metabolism of amino acids. All five of these enzymes have been isolated from different sources and characterized (*1–7*).

With respect to the reaction mechanism, α,β-dehydrogenation catalyzed by these enzymes is unique in that it involves the rupture of two kinetically stable hydrogen bonds (*8*). Its mechanism is also of considerable chemical interest, since it has not yet been elucidated in detail. The pioneering work in the study of α,β-dehydrogenation was done by Beinert and his co-workers, who, almost 30 years ago, purified an MCAD to apparent homogeneity, and made the first proposal on its mechanism (*9, 10*). Although their proposal has been overlooked, it has been proven correct and was verified by our recent work (*11*).

SPECTRAL PROPERTIES OF ACYL-CoA DEHYDROGENASES

These flavin enzymes are peculiar in that some of them can be isolated either in a green or in a yellow form. Some representative spectra are shown in Fig. 1.

The green form is characterized by an absorption maximum around 710 nm (*13*) in addition to the ~450 nm band of the oxidized

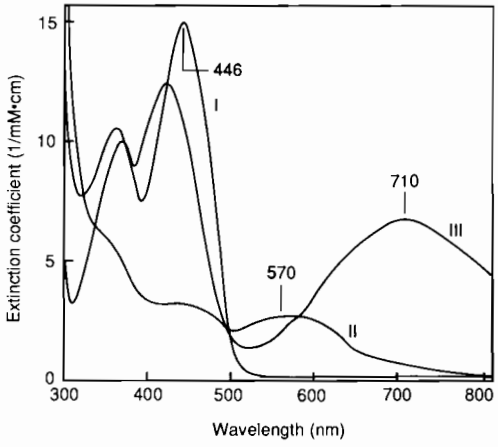


Fig. 1. Absorption spectra of some forms of acyl-CoA dehydrogenases. I, MCAD purified from pig kidney; II, I+octanoyl-CoA; III, *Megasphera elsdenii* butyryl-CoA dehydrogenase+CoA-SS$^-$ (*12*).

flavin chromophore. The "green color" is always associated with very tightly bound CoA derivatives. In the case of the bacterial butyryl-CoA dehydrogenase, it is due to the interaction of a CoA persulfide with the oxidized flavin, which gives rise to the "green absorption" (*12*). This phenomenon most probably is ascribable to a charge transfer interaction (*14*) between the persulfide anion as donor and the oxidized flavin as acceptor. Normal substrates can also induce the appearance of a green color upon reaction with the oxidized enzyme. In this case, the absorption, which has a broad absorption band extending beyond 600 nm and has different maxima depending on the substrates (*15*), results from a charge transfer interaction between reduced enzyme flavin and enoyl-CoA, the product of oxidation of saturated acyl-CoA.

CATALYTIC FEATURES AND SOME UNUSUAL KINETIC PROPERTIES

The reaction catalyzed by these dehydrogenases yields enoyl-CoA from acyl-CoA by removal of the proR, proR hydrogens at positions 2 and 3 (*16, 17*) as represented by the following equation:

$$R\text{-}CH_2\text{-}CH_2\text{-}CO\text{-}SCoA \rightleftharpoons R\text{-}CH{=}CH\text{-}CO\text{-}SCoA + 2H^+ + 2e^-$$

The second product is formally a hydride equivalent, which is transferred to the oxidized flavin (*18*). These redox equivalents are subsequently transferred to the respiratory chain *via* "electron transferring flavoprotein" (ETF) and ETF-ubiquinone oxidoreductase (*19, 20*). These latter two proteins also contain FAD as a cofactor. They serve in the transfer of electrons (one at a time) originating from β-oxidation and from other sources, and probably also in adjustment of the redox potential.

The interplay of the different enzymes involved in the β-oxidation and the metabolic pathway are outlined in Fig. 2.

Of particular interest from a kinetic point of view is the time course of the reaction of acyl-CoA with the oxidized enzyme. Upon mixing of the reactants, a Michaelis complex is formed (*11, 21*), which is reflected by the perturbation of the spectrum of the oxidized enzyme flavin (*cf.* Fig. 1). Subsequently a decrease of this absorption ensues, which proceeds in two distinct phases (the absorption decrease can be triphasic, but the third phase will not be addressed here, since it probably does not play a role in catalysis (*11, 21*)). This leads to a

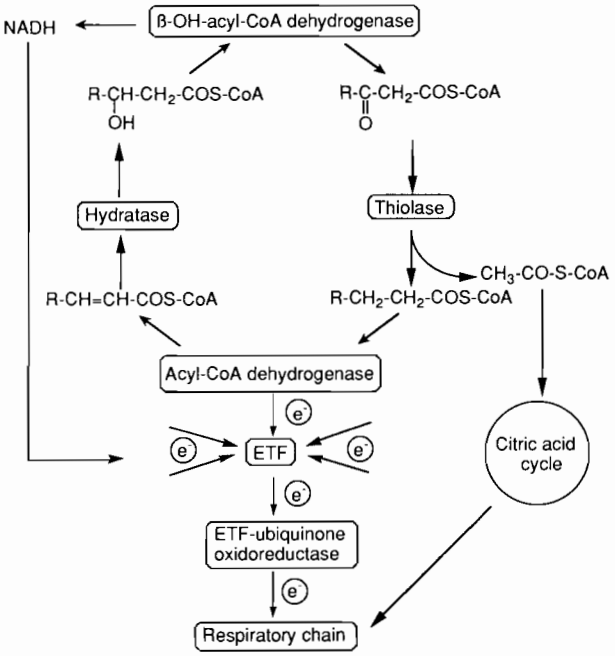


Fig. 2. Acyl-CoA derivatives of the β-oxidation cycle, enzymes involved, and interplay of different enzymes and metabolic pathways. ETF accepts redox equivalents also from other sources.

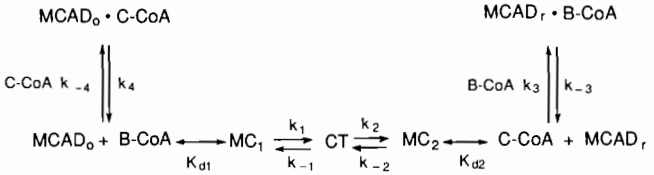
decrease in the 450 nm absorption of the oxidized enzyme to an extent (40% to almost 100%), which is dependent on the nature of the acyl-CoA substrate, in particular on its chain length and on the redox potential of the flavin cofactor (*22*). This multiphasic kinetic behavior has led to most disparate interpretations such as half-site reactivity (*23*), enzyme heterogeneity, or occurrence of exotic intermediates (*24*). As was shown recently (*11*), the mechanism underlying reduction is rather straightforward and can be accounted for by the minimal sequence of Scheme 1. The values of the steps of Scheme 1 are listed in Table I.

They have either been measured directly or obtained from simulations (*11*). The biphasic reduction behavior and the "incomplete reduction" arise from the following combination of rates:

i) Binding of substrate to the oxidized enzyme is very tight (K_{d1}), but somewhat less than that of the product to reduced enzyme (K_{d2}).

ii) "CT" is a complex consisting of reduced flavin and enoyl-CoA, which has the charge transfer absorption mentioned above, but which is different from the Michaelis complex between reduced enzyme and crotonyl-CoA (MC_2). What makes the difference between these two species is not known, but it might be due to a different state of ionization of a protein function or to differences in protein conformation.

iii) The steps k_1 and k_{-1} have almost identical values in the case of butyryl-CoA, *i.e.*, MC_1 and CT exist in a $\sim 1:1$ equilibrium. Since the chromophore in MC_1 is oxidized flavin and in CT reduced flavin, and since these species are the major constituents of the system during



Scheme 1. Kinetic scheme proposed for the reaction of MCAD from pig kidney with butyryl-CoA. The symbols stand for the following: $MCAD_o$, oxidized enzyme; B-CoA, butyryl-CoA; MC_1, Michaelis complex formed *via* step K_{d1}; CT, charge transfer complex between reduced enzyme flavin and crotonyl-CoA; MC_2, Michaelis complex between reduced enzyme and crotonyl-CoA; C-CoA, crotonyl-CoA; k, single interconversion steps; K, rapid equilibrium steps (*11*).

TABLE I

Rate and Equilibrium Constants for the Steps in the Reaction of MCAD from Pig Kidney and Butyryl-CoA

Parameter	Measured value	(Best fit to simulation)
k_1 (sec^{-1})	1.7	1.9
k_{-1} (sec^{-1})	0.6	0.45
k_2 (sec^{-1})	0.07	0.07
k_{-2} (sec^{-1})	220	450
k_3 ($M\ sec^{-1}$)	3×10^6	3×10^6
k_{-3} (sec^{-1})	0.15	0.20
k_4 (sec^{-1})	20	16
k_{-4} ($M\ sec^{-1}$)	9.8×10^{-5}	1.2×10^{-6}
K_{d1} (μM)	37	80
K_{d2} (μM)	(upper limit: 300)	20

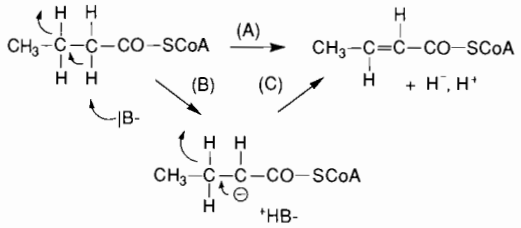
For the identification of the single steps see Scheme 1 (*11*).

the first half of the reaction, this equilibrium can be approached by the "half reaction" or the half reduction mentioned above.

iv) The biphasic kinetics of reduction is explained as follows: a) The first rapid phase corresponds to reduction and approach to the equilibrium $MC_1 \rightleftharpoons CT$, and b) the second (slower) phase is governed by the rate of dissociation of crotonyl-CoA from MC_2, which leads to the approach to a new, overall equilibrium situation in which excess substrate butyryl-CoA binds to reduced enzyme (steps k_3, k_{-3}), and free product (crotonyl-CoA) binds to oxidized enzyme (steps k_4, k_{-4}). This kinetic scheme is essentially an expansion of the original one put forward by Beinert and co-workers (*9, 10*), and its validity has been supported by extensive simulations of the reactions monitored by use of normal as well as various deuterated butyryl-CoAs (*11*).

The isotope effects observed with deuterated substrates are of particular mechanistic importance. With α-dideutero-, β-dideutero-, and α,β-tetradeutero-butyryl-CoA, the rate of flavin reduction is reduced by the factors ~ 2, ~ 15, and ~ 30, respectively (*21*). Thus, the isotope effect observed with the α,β-tetradeuterated substrate is approximately the multiple of those observed with either α- or β-dideutero compounds. This has been interpreted as being strong evidence that the C(α)-H and the C(β)-H bonds are cleaved in a concerted fashion (step A, Scheme 2) as opposed to a two-step reaction for which an addition of the single isotope effects might be expected (*15*). A synchronous reaction appears unlikely in view of the different polarization of the two C-H bonds to be cleaved, and of the different modes by which this occurs. A two-step mechanism would proceed over the intermediate carbanion (step B) to yield the same products *via* the subsequent step C shown in Scheme 2.

It should be pointed out, however, that the enzyme also catalyzes the formation of a definite α-carbanion (step B), as is demonstrated by the occurrence of exchange of the α-hydrogen in aqueous solvent (*18*). This occurs when the reaction cannot proceed to completion, *e.g.*, when no β-hydrogen is present (when step C is impossible), or might also occur as a "side reaction" accompanying normal dehydrogenation (*18*) (*cf.* also the next section). It should be noted that the occurrence of carbanion formation does not exclude a concerted mechanism for the rupture of both C-H bonds in α,β-dehydrogenation.

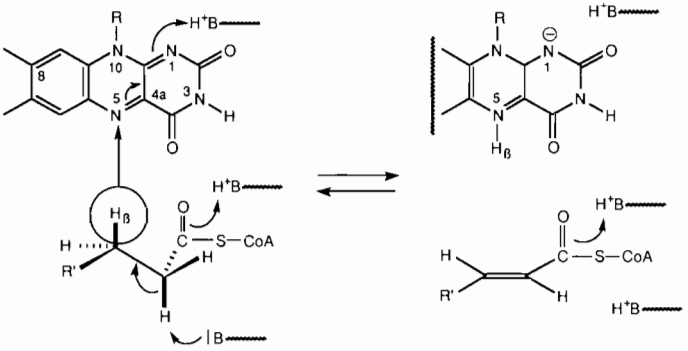


Scheme 2. Concerted or synchronous (step A) *versus* two-step (B+C) mechanism for α,β-dehydrogenation of acyl-CoA substrates.

MECHANISM OF α,β-DEHYDROGENATION

The α,β-dehydrogenation reaction is more complicated than most other dehydrogenations in that it involves the rupture of two C-H hydrogen bonds, as opposed to one in the usual case. Scheme 3 depicts the relative orientation of substrate and flavin, and the possible roles of amino acid functional groups at the active center that can be proposed to be present at the active center of acyl-CoA dehydrogenases.

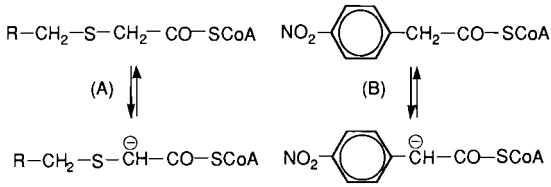
Cornforth, in the early fifties, was the first to propose a mechanism initiated by abstraction of the α-hydrogen as a proton (*25*). For such a process the base functioning in proton abstraction is of eminent importance. Using mechanism-based inactivators, which covalently modify the protein (*cf.* the later section), Fendrich and Abeles (*26*), and later Powell and Thorpe (*27*), identified a glutamic acid residue, and proposed it to be this proton-abstracting base. Powell and Thorpe (*27*) also determined the sequence of the amino acids around this residue, and by comparison with the full sequence (Table II), it turns out that it is located at position 376 towards the C-terminal of the protein. Most recently, using site-directed mutagenesis, we replaced Glu376 with glutamine, and found that this leads to essentially complete loss of MCAD activity upon expression in *Escherichia coli* (*28*). This provides strong support for a crucial role of Glu376 in catalysis. If Glu376 is not the proton-abstracting base itself, it could form a relay system with a histidine, for example, as has been found in several cases. In the second eventuality, however, it would be difficult to envisage why it is the carboxylate, and not the other function (*e.g.*, histidine imidazol), that reacts nucleophilically with the allene.



Scheme 3. Stereochemistry and mechanism of α,β-dehydrogenation catalyzed by flavin-dependent acyl-CoA dehydrogenases. R, ribose-P-AMP; R′, alkyl.

The mode of transfer of redox equivalents to the oxidized flavin is likely to occur as shown in Scheme 3. Note that breaking of the C(α)-H and C(β)-H bonds is *trans* (proR, proR), that this occurs in a concerted fashion, and that the Hβ is transferred directly to the oxidized flavin position N(5). Evidence for the latter comes from studies using 5-deaza-FAD reconstituted MCAD in which the exchange of hydrogens at position N(5) does not occur (*18*). The hydride is introduced onto the flavin Re-side (*29*), *i.e.*, from below the plane in the drawing of Scheme 3.

As is the case with L-lactate oxidase (*30*), glycollate oxidase (*31*), and cytochrome b_2 (*32*), it is proposed that a positive charge or dipole is placed near position N(1)-C(2)=O in order to facilitate uptake of the negative charge associated with the hydride, and its stabilization in the reduced form ("charge sink"). Also, it is most likely that a further function, which could be either a strong hydrogen bridge or a positively charged group (or both), interacts with the -COS- moiety of CoA esters as shown. The effect would be to stabilize transiently, *e.g.*, *via* an inductive effect, the incipient negative charge resulting from α-proton abstraction (see Scheme 2). Indeed, Thorpe has provided support for the suggestion that substrate β-thia-analogs (Scheme 4, A) exist in their anionic forms at the enzyme active center (*33*). This would suggest that the pK of the C(α)-H is lowered considerably, probably by at least 7–8 units upon binding to the active center. In line with this, we have found that p-NO_2-phenyl-acetyl-CoA (Scheme 4, B) is also bound as the anionic species (*34*). Binding induces the appearance of a long wave-

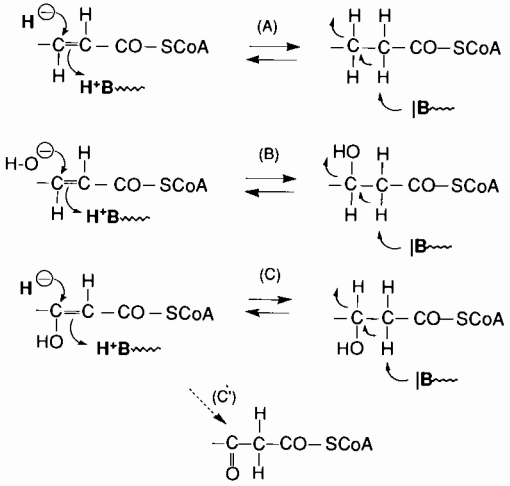


Scheme 4. Ionization of "acidic" substrate analogs upon binding to acyl-CoA dehydrogenases. Upper form, free state; lower form, bound to the enzyme. Note that the negative charge will be localized extensively in both cases.

length charge transfer band (maximum ~720 nm) typical for the presence of a good electron donor, and of a strong absorption in the 450–500 nm region, which can hardly be attributed to the oxidized flavin, and which thus belongs to the anionized *p*-NO_2-phenyl-acetyl moiety itself (*34*).

ADDITIONAL CATALYTIC PROPERTIES OF ACYL-CoA DEHYDROGENASES

It was noted quite early (*35*), that hydratase (crotonase) activities accompanied acyl-CoA dehydrogenases during purification. Such activities have complicated the study of the reaction course and of the products of α,β-dehydrogenation considerably, and several attempts to eliminate them from typical preparations of bacterial SCAD or MCAD were not successful. It was thus realized that MCAD and SCAD intrinsically possess the capacity to hydrate the double bond of enoyl-CoA. While the rate of this reaction is slow, it is much superior to that of the uncatalyzed one. The rate is ~1/500 that of the dehydrogenation of butyryl-CoA in the case of the enzyme from pig kidney (*36*). The same enzyme has also been reported to catalyze the dehydrogenation of β-OH-acyl-CoA analogs (*37*), this reaction being very slow. From a mechanistic point of view, comparison of the three activities of this enzyme as done in Scheme 5 is of considerable interest. It suggests that the same active center machinery is basically able to catalyze different types of reactions that superficially bear little resemblance. On the other hand, this problem can be viewed from an opposite direction and taken as suggesting that elimination of HO^- and of H^- are mechanistically similar, the acceptors being H^+ and oxidized flavin, respectively. In Scheme 5, this would be the sets of reactions proceeding from right to



Scheme 5. Comparison of α,β-dehydrogenation, β-hydratase activity, and β-OH-dehydrogenation catalyzed by pig kidney acyl-CoA dehydrogenase.

left. Thus in the case of both α,β-dehydrogenation and hydration (reactions A and B in Scheme 5), the same base catalyzes donation of a proton to a double bond. Note that the "reverse" of the normal reaction (*i.e.*, the hydrogenation of a double bond) is depicted in this scheme for comparison. An ancestral enzyme capable of binding CoA-derivatives might have evolved to optimize each single type of reaction, *i.e.*, hydration and α,β-dehydrogenation. It is conceivable that the extent of hydration in MCAD is governed by the availability and accessibility of water at the active center, and by its positioning in relation to the double bond of the enoyl moiety. The occurrence of β-OH-dehydrogenation is not entirely surprising, since it corresponds to a normal α,β-dehydrogenation with the difference that an enolate is formed first (step C), which subsequently tautomerizes *via* step C′ to the final product β-keto-acyl-CoA. Acyl-CoA dehydrogenases can thus catalyze the three first steps of β-oxidation. We would like to stress, however, that these "side activities" most probably have no physiological significance and that they are merely of mechanistic interest.

BIOSYNTHESIS AND SEQUENCE

As with most proteins imported into mitochondria, MCAD is

produced with a leader sequence and with a molecular weight some 2,900 Da larger than that of the mature protein (*38, 39*). The sequences of cDNAs obtained from mRNAs from rat liver (*39, 40*), from human liver and placenta (*41, 42*), and from pig liver (*43*) have been determined and are compared in Table II. Unfortunately, clones encoding the pig kidney enzyme, probably the one about which most mechanistic and biochemical information exists, have not yet been obtained, and only peptides accounting for approx. 70 amino acids have been sequenced (*27, 44, 45*). In the case of the rat liver MCAD the N-terminus appears to be free, while in the case of the pig kidney and pig liver enzymes, it is blocked (*44, 45*). Nevertheless, an N-terminal sequence of the pig liver MCAD could be obtained since the enzyme undergoes partial proteolysis at the N-terminal end during purification (*46*). The N-terminal sequence shown in Table II for the pig kidney enzyme was obtained upon treatment of the denatured enzyme with acylamino acid-releasing enzyme (Takara Shuzo Co., Kyoto). However, this procedure demonstrated an extremely poor performance with this enzyme in our hands, and thus we must stress that the sequence shown is tentative.

Among the rat liver and human liver and placenta sequences the homology is ~88%. The pig liver and the pig kidney enzymes are also highly homologous, as far as can be deduced from the present data. They are, however, not identical, the differences residing mainly in the N-terminal region (Table II). This suggests that they are different, although closely related, proteins (isozymes) probably encoded by different genes. LCAD and SCAD also have been sequenced recently (*47*), and their sequences are similar to the MCAD ones. This suggests that most acyl-CoA dehydrogenases belong, not unexpectedly, to a class of homologous enzymes and that they are phylogenetically closely related. The active site encompasses the C-terminal region of the peptide (*48*), the glutamic acid identified by Powell and Thorpe for the pig kidney enzyme (*27*) being at position 376 (Table II).

EXPRESSION AND ACTIVE SITE-DIRECTED MUTAGENESIS

The cDNAs encoding human liver (*41*) and rat liver (*40*) MCAD have been introduced into inducible expression plasmids.

First, *E. coli* cells were transformed with human liver MCAD cDNA and MCAD protein was expressed in active form at 0.2–0.4% of

TABLE II

Comparison of Amino Acid Sequences of MCAD from Rat Liver (Ra Li), Human Liver (Hu Li), Pig Liver (Pi Li), and Pig Kidney (Pi Ki)

```
Ra  Li                  (precursor sequence)          MAAALRRGYKVLRSVSHFECRAQHT
                                                      :::   :   :::: : :  : :::
Hu  Li                  (precursor sequence)          MAAGFGRCCRVLRSISRFHWRSQHT
                                                            :::: : : ::::::
Pi  Li                  (precursor sequence)                EFRQVLRSLSHFGWRSQHT
Pi  Ki _____________    (precursor sequence not known)
N°      12345678901234567890123456789012345678901234567890123456789012345678901234567890
                  1         2         3         4         5         6         7

Ra  Li KPSLKEEPGLGFSFELTEQQKEFQTIARKFAREEIIPVAPDYDKSGEYPVPLIKRAWELGLINTHIPESC
       :     :::::::::.:::::::::  :::::::::::::: .::::.:::::::::.:::::::.:::::: :
Hu  Li KANRQREPGLGFSFEFTEQQKEFQATARKFAREEIIPVAAEYDKTGEYPVPLIRRAWELGLMNTHIPENC
       ::  : ::: ::::: :::::::::::::::::::::::::::::::::.:::::::::..:::::::::::::::
Pi  Li KAVPQCEPGSGFSFELTEQQKEFQATARKFAREEIIPVAAEYDRTGEYPVPLLKRAWELGLMNTHIPESF
          ::  : :                          ::::::::::::
Pi  Ki .KVPNSEGG.....................EEIIPVAAEYDR.....................

Ra  Li GGLGLGTFDACLITEELAYGCTGVQTAIEANSLGQMPVIIAGNDQQKKKYLGRMTEQPMMCAYCVTEPSA
       ::::::::::::::.:::::::::::::::: ::::::::.:::::::::::::::::::: : :::::::: :
Hu  Li GGLGLGTFDACLISEELAYGCTGVQTAIEGNSLGQMPIIIAGNDQQKKKYLGRMTEEPLMCAYCVTEPGA
       :::::: .: :::.:::::::::::::::: :.:::.:.:: :: :: :::::::::::::::::::::::::
Pi  Li GGLGLGIIDSCLITEELAYGCTGVQTAIEANTLGQVPLIIGGNYQQQKKYLGRMTEEPLMCAYCVTEPGA
                                                                 ::::::::::::::::
Pi  Ki ..................................................MTEEPLMCAYCVTEPGA

Ra  Li GSDVAGIKTKAEKKGDEYVINGQKMWITNGGKANWYFVLTRSNPDPKVPASKAFTGFIVEADTPGIHIGK
       ::::::::::::::::::.:::::::::::::::.: :: :::: :: ::::::::::::::: ::.
Hu  Li GSDVAGIKTKAEKKGDEYIINGQKMWITNGGKANWYFLLARSDPDPKAPANKAFTGFIVEADTPGIQIGR
       :::::::::::::::::::::::::::::::::::::::::::::::::::: ::::::::::::::.::::
Pi  Li GSDVAGIKTKAEKKGDEYIINGQKMWITNGGKANWYFLLARSDPDPKAPASKAFTGFIVEADTPGVQIGR
       :::             :::::: :
Pi  Ki GSD...........GDEYIIAGS.............................................

Ra  Li KELNMGQRCSDTRGITFEDVRVPKENVLIGEGAGFKIAMGAFDRTRPTVAAGAVGLAQRALDEATKYALD
       ::::::::::::::: ::::.:::::::::.:::::.::::::.::: ::::::::::::::::::::::.
Hu  Li KELNMGQRCSDTRGIVFEDVKVPKENVLIGDGAGFKVAMGAFDKTRPVVAAGAVGLAQRALDEATKYALE
       ::.:.:::::::::::::::.::::::: :.:::::.::: :::::: ::::::::::::::::::::::::
Pi  Li KEINIGQRCSDTRGIVFEDVRVPKENVLTGEGAGFKIAMGTFDKTRPPVAAGAVGLAQRALDEATKYALE
                                                  ::::::::
Pi  Ki .....................................IAMGTFDK.........................

Ra  Li RKTFGKLLVEHQGVSFLLAEMAMKVELARLSYQRAAWEVDSGRRNTYFASIAKAFAGDIANQLATDAVQI
       ::::::::::::.: .::.:::::::::::::.:::::::::::::::::::.:::::::::::::::::::
Hu  Li RKTFGKLLVEHQAISFMLAEMAMKVELARMSYQRAAWEVDSGRRNTYYASIAKAFAGDIANQLATDAVQI
       :::::::: ::: :::.::.:::::::::.::::::::.:::::::::::::::.: ::::::::::::::.
Pi  Li RKTFGKLLAEHQGISFLLADMAMKVELARLSYQRAAWEIDSGRRNTYYASIAKAYAADIANQLATDAVQV

Pi  Ki ..........................................................................

Ra  Li FGGYGFNTEYPVEKLMRDAKIYQIYEGTAQIQRLIIAREHIEKYKN
       .:: :::::::::::::::::::::::::: :::::.:::::.:::::
Hu  Li LGGNGFNTEYPVEKLMRDAKIYQIYEGTSQIQRLIVAREHIDKYKN      (:) = identical amino acids;
       .:::::::::::::::::::::::::::::: ::::.:.::::: .:::
Pi  Li FGGNGFNTEYPVEKLMRDAKIYQIYEGTAQIQRIIIAREHIGRYKN      (.) = conservative exchange
                                 ::::::::::::::
Pi  Ki .....................IYQIYEGTAQIQR.............
```

Amino acid sequence of human placenta MCAD is the same as that of human liver.

the total soluble protein. To confirm that Glu-376 is the central residue, the cDNA for human liver MCAD was modified by genetic engineering, the amino acid Glu-376 was changed to Gln-376, and the modified protein directed by this cDNA was synthesized in *E. coli* (*28*). The corresponding extracts showed no detectable MCAD activity, although the modified MCAD protein was detectable by SDS-PAGE/immunoblotting analysis. This result confirmed the identification of Glu-376 as a crucial residue.

Second, N-terminus-different rat liver MCADs were synthesized in *E. coli* carrying expression plasmids differing in their 5′-region (*40*). pRMCADm-1, 2, and 3 contained the sequence for the last 15, 9, and 2 amino acid residues, respectively, of the leader peptide, and pRMCADm-4, 5, and 6 lacked that for 3, 26, and 42 amino acid residues of the N-terminal portion of the mature enzyme. Relative MCAD activity of the corresponding extracts was 0, 60, 100, 90, <5, and 0% for the protein directed by pRMACDm-1, 2, 3, 4, 5, and 6, respectively. MCAD purified to homogeneity from pRMCADm-3-carrying *E. coli* had almost the same properties as the rat liver mitochondrial enzyme, *e.g.*, absorption spectrum, specific activity, and kinetic parameters; whereas proteins purified from pRMCADm-1- and -6- bearing *E. coli* did not contain FAD. These findings indicate that the leader peptide alters the conformation of the apoprotein and/or disturbs the FAD binding, and that the deletion of the N-terminal region of the apoprotein to some extent alters the conformation of the apoprotein, which results in its inability to bind the flavin coenzyme.

THREE DIMENSIONAL STRUCTURE

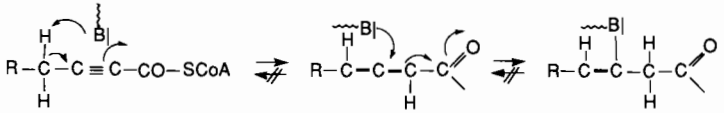
The X-ray crystallographic structure of MCAD purified from pig liver has been reported recently at a resolution of ~ 3 Å by Kim and Wu (*48*). The enzyme is a tetramer containing one FAD cofactor in each subunit. The polypeptide chain is folded into three main domains termed the N-terminal and the C-terminal domains, which are composed of α-helices, and an α,β-sheet domain in the intervening region. Although the amino acid side chains have not yet been identified, several interesting features emerge from the schematic backbone drawing. The FAD molecule is bound in an extended fashion, as found with most other flavoproteins. The domain around the isoalloxazine moiety of FAD, which encompasses the active site, is different from that found

in other flavin-dependent enzymes, and it has been suggested that MCAD might belong to a structural family not previously encountered among flavoproteins (*48*). The binding site for the CoA moiety also has been localized (*49*).

SUBSTRATE ANALOGS AND ARTIFICIAL INHIBITORS

Essentially any acyl-CoA molecule which is not a substrate, will be a competitive inhibitor, since binding energy appears to result to a large extent from the interaction of the protein with the CoA moiety (*50*). Thus enoyl-CoA products, β-OH-acyl-CoAs, and especially β-keto-acyl-CoAs, *i.e.*, the three products following saturated acyl-CoAs in the β-oxidation cycle, are all inhibitors. It is thus conceivable that all of them play a role in regulation of the enzyme reaction *in vivo*.

There has been quite widespread use of modified substrates and of analogs for mechanistic studies, with most relevant mechanistic information having resulted from their use. A particular class of analogs is characterized by their conversion into products having distinctly different absorption spectra. This has been used for monitoring the course of the dehydrogenation reaction directly and for comparing it to the course of flavin reduction (*51, 52*). Analogs containing either the allenic (*53*) or the acetylenic function (*27*) in position α and β have turned out to be efficient mechanism-based inhibitors (*8*). Their mode of action is derived from the mechanism depicted in Schemes 2 and 3, a typical case being that of the α-alkynoic derivatives, which are converted to allenic species *via* tautomerization as shown in Scheme 6 (*27*). The allene undergoes a Michael addition, probably by the same base that served in proton abstraction, this being facilitated by a function interacting with the -COS-carbonyl (*cf.* also Scheme 3). Glutamic acid 376 was identified by analysis of the product obtained from this reaction (*27*).

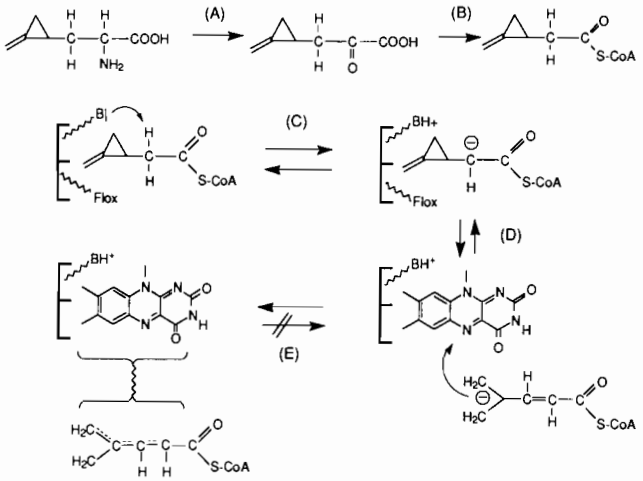


Scheme 6. Mode of enzyme inactivation by acyl-CoA analogs containing α-alkyne substituents.

NATURALLY OCCURRING INHIBITORS

Hypoglycine (structure top left, Scheme 7) is the case of a particular inhibitor of β-oxidation that has achieved some celebrity over the last forty years (*54*). It is an unusual amino acid found in the unripe ackee fruit (*Blighia sapida*) and in similar plants, and induces strong hypoglycaemia and often death in mammals (*55*). Hypoglycine is first transaminated *in vivo* to the α-keto acid (step A, Scheme 7), which is then converted to methylenecyclopropylacetyl-CoA (step B). This CoA conjugate is clearly a substrate analog in that it is recognized by MCAD, SCAD, and probably also by isovaleryl-CoA dehydrogenase, which are inactivated irreversibly by this conjugate (*56*). The mechanism of this process is not known in detail, although it has been established that the flavin coenzyme becomes covalently modified during inactivation (*56*). Most likely, abstraction of the α-proton generates a carbanion that is unlikely to react further *via* dehydrogenation. This carbanion, or alternatively a radical resulting from transfer of one electron to the flavin, undergoes a ring-opening process D, and the resulting species binds covalently to the flavin (step E).

Analysis of the products obtained upon denaturation of the



Scheme 7. Mode of transformation of hypoglycine into methylenecyclopropylacetyl-CoA and irreversible inactivation of MCAD by the latter.

protein reveals up to 6 different chromophores, all of which are derived from the flavin. One of them probably results from addition of the inactivator to the flavin at position 6. The second one is a modified 4a, 5-dihydroflavin in which the inactivator is probably linked to both the C(4a) and the N(5) of the flavin to form a cyclic adduct (*57*). The exact structure of these products is still under investigation.

GENETIC DEFECTS

In the past few years, inherited genetic defects involving acyl-CoA dehydrogenases have been recognized as being the origin of some cases of sudden infant death syndrome (*58*). Thus, according to Allison *et al.* (*59*), 2% of infants who died showing the typical sudden infant death syndrome were deficient in MCAD activity, and the incidence of genetic defects involving this enzyme was estimated as ~0.02% of births. In some cases, the molecular defects underlying this syndrome have been studied (*58*), and it seems that in most cases MCAD deficiency results from point mutations. A particularly interesting case is that reported by Strauss and collaborators (*60*), who found that in a specific patient, expression of mRNA encoding MCAD was normal, while the mature MCAD protein was essentially absent. Inagaki *et al.* (*42*) also found the same phenomenon. One of the two patients examined was found to lack the MCAD protein; whereas another patient had the enzyme protein but it was not catalytically active. The levels of mRNA for MCAD in these two patients were almost the same as the level of the control subject.

CONCLUSION AND OUTLOOK

After a long period of neglect, enzymes catalyzing transformations of acyl-CoA substrates, in particular the different acyl-CoA dehydrogenases, have returned into the focus of active research. The biochemical properties of MCAD, especially those of the pig kidney and pig liver enzymes, have been extensively studied. Thus we now have quite a detailed understanding of the kinetic behavior of MCAD, and major aspects of the chemical mechanism have been elucidated. The recognition of the importance of genetic defects affecting α,β-dehydrogenation has led to the cloning and sequencing of several dehydrogenases (*cf.* Table II), and it is expected that the sequences of

"long chain", "short chain", and "branched chain" dehydrogenases will be reported shortly (*58*). Similarly the three dimensional structure of a MCAD at a resolution allowing the identification of the side chains of the polypeptide is expected to be accomplished soon (*60*). The combination of such information with that resulting from active site-directed mutagenesis will help us to elucidate the molecular mechanism of α,β-dehydrogenation, and also to understand the molecular basis of inherited disorders.

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