Mechanism-Based Inhibitor Discrimination in the Acyl-CoA Dehydrogenases†

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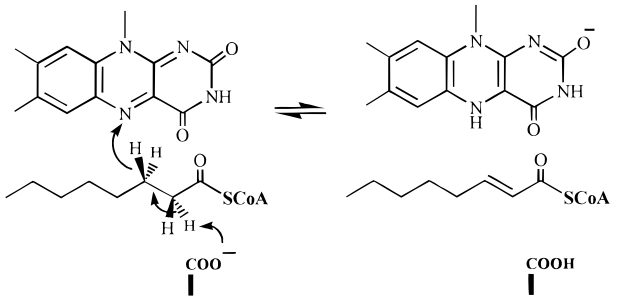
ABSTRACT: The catalytically essential glutamate base in the acyl-CoA dehydrogenase family is found either on the loop between J and K helices (e.g., in short-chain, medium-chain, and glutaryl-CoA dehydrogenases) or on the G helix (long-chain and isovaleryl-CoA dehydrogenases). While active-site bases at either position are functionally equivalent with respect to α-proton abstraction, reactions that require removal of a γ-proton show marked differences between the two enzyme classes. Thus short-chain, medium-chain, and glutaryl-CoA dehydrogenase are rapidly inactivated by 2-pentynoyl-CoA with abstraction of a γ-proton, whereas isovaleryl-CoA dehydrogenase is not significantly inhibited. This resistance is not due to weak binding: the complex between isovaleryl-CoA dehydrogenase and 2-pentynoyl-CoA shows a K_d of 1.8 μM at pH 7.6. Migration of the catalytic base to the loop between J and K helices (using the Glu254Gly/Ala375Glu double mutant) makes isovaleryl-CoA dehydrogenase sensitive to irreversible inhibition by 2-pentynoyl-CoA. Molecular modeling suggests that this mutation brings the catalytic base close enough to abstract a γ-proton from the bound inhibitor. Experiments with two mechanism-based inactivators that target the FAD of the medium- and short-chain acyl-CoA dehydrogenases support this conclusion. 3-Methyl-3-butenoyl-CoA requires activation by α-proton abstraction and rapidly yields a reduced flavin adduct with wild-type isovaleryl-CoA dehydrogenase. In contrast, the isomeric 3-methyl-2-butenoyl-CoA is inert toward this enzyme because it cannot be activated by γ-proton abstraction. Molecular modeling supports these observations. This unusual selectivity toward mechanism-based inactivators provides additional discrimination between members of the acyl-CoA dehydrogenase family.

Substrate activation in the acyl-CoA dehydrogenases (Beinert, 1963; Engel, 1992; Ghisla et al., 1994; Thorpe & Kim, 1995) involves abstraction of the *pro-R* α-proton with elimination of the *pro-R* β-hydrogen as a hydride equivalent to the N-5 position of the flavin ring (Scheme 1; Biellman & Hirth, 1970; Frerman et al., 1980; Fendrich & Abeles, 1982; Ghisla et al., 1984). Reoxidation of the two-electron-reduced flavin by electron-transferring flavoprotein completes catalysis (Beinert, 1963). The catalytic base depicted in Scheme 1 was suggested to be Glu376 in the medium-chain acyl-CoA dehydrogenase because it was the target of the mechanism-based inactivator 2-octynoyl-CoA (Chart 1; Powell & Thorpe, 1988). This tentative assignment was confirmed with elucidation of the crystal structure of the medium-chain dehydrogenase (Kim & Wu, 1988; Kim et al., 1993) and by mutagenesis (Bross et al., 1990). A striking aspect of the acyl-CoA dehydrogenase family is that this catalytically essential residue is not conserved (Kelly et al., 1987; Tanaka & Indo, 1992; Kim et al., 1992; Djordjevic et al., 1994; Mohsen et al., 1995a; Thorpe & Kim, 1995; Nandy et al., 1996b; Lee et al., 1996): Glu376 is a Gly or Ala residue in the long-chain or isovaleryl-CoA dehydrogenases and the base is Glu261 or Glu254, respectively. These assignments, initially suggested by homology modeling of

Scheme 1



the structures of LCAD[1] and IVAD (Kim et al., 1992), were confirmed by subsequent mutagenesis studies (Djordjevik et al., 1994; Mohsen & Vockley, 1995a). Finally, double mutants have been constructed in both MCAD (Thr255Glu/Glu376Gly; Ghisla et al., 1994; Nandy et al., 1996a,b; Lee et al., 1996) and IVAD (Glu254Gly/Ala375Glu; Mohsen & Vockley, 1995a) to move the catalytic base between polypeptide segments. These constructs retain a significant fraction of wild-type activity (Ghisla et al., 1994; Nandy et al., 1996b; Lee et al., 1996; Mohsen & Vockley, 1995a). Thus, placement of the base at either position allows the carboxylate to abstract an α-proton from bound acyl-CoA substrates (see later).

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[1] Abbreviations: DCIP, 2,6-dichlorophenolindophenol; PMS, phenazine methosulfate; SCAD, short-chain acyl-CoA dehydrogenase; dm-IVAD, Glu254Gly/Ala375Glu double mutant of isovaleryl-CoA dehydrogenase; MCAD, medium-chain acyl-CoA dehydrogenase; BBCD, bacterial butyryl-CoA dehydrogenase; LCAD, long-chain acyl-CoA dehydrogenase.

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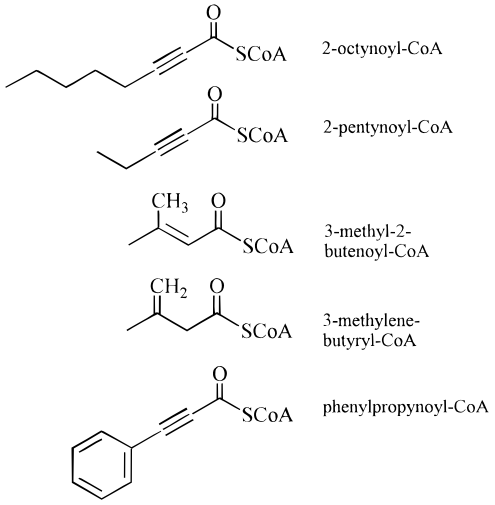
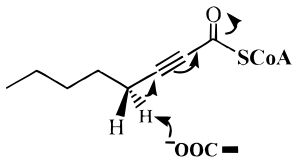
Chart 1



Scheme 2

Scheme 3



In addition to normal catalysis, the bacterial short-chain acyl-CoA dehydrogenase can remove a proton from C-2 of 3-butenoylpantetheine [(vinylacetyl)pantetheine] and reprotonate at C-4 to give the thermodynamically favored crotonylpantetheine (Fendrich & Abeles, 1982; Scheme 2). The same isomerase reaction using CoA thioesters was observed with the medium-chain dehydrogenase (Powell, 1988). In reverse, 3,4-pentadienoyl-CoA is isomerized to the 2,4-diene (Wenz et al., 1985). Similarly, abstraction of a proton at C-4 occurs during activation of the mechanism-based inhibitor 2-octynoyl-CoA (Freund et al., 1985; Powell & Thorpe, 1988; Scheme 3), eventually leading to covalent modification of Glu376. The short-chain dehydrogenases are similarly sensitive to inhibition by 2-pentynoyl- or 2-butynoyl-CoA (Lundberg & Thorpe, 1993; Dakoji et al., 1996). Recent work by Liu, Stankovich, and colleagues has confirmed that the residue depicted in Scheme 3 (Powell & Thorpe, 1988; Nandy et al., 1996a,b) is indeed the catalytic base (Dakoji et al., 1996).

In contrast to the short- and medium-chain enzymes, preliminary studies with isovaleryl-CoA dehydrogenase (Lundberg & Thorpe, 1993) and the beef liver long-chain dehydrogenase (Ankele et al., 1991; Nandy et al., 1996b) suggest that these enzymes are not irreversibly inactivated by 2-alkynoyl-CoA inhibitors. The present paper shows that isovaleryl-CoA dehydrogenase fails to react with two widely different inhibitors, probably because the active-site base cannot reach the C-4 position of the bound thioester. Indeed, a comparison of the behavior of isovaleryl-CoA dehydrogenase and the short-chain enzyme reveals a surprising selectivity of behavior toward mechanism-based inactivators. Such differences might be exploited in the design of more discriminating inhibitors of the acyl-CoA dehydrogenase family.

MATERIALS AND METHODS

Materials. Short-chain acyl-CoA dehydrogenase was purified from porcine liver mitochondria (Shaw & Engel, 1984; Lundberg & Thorpe, 1993). Recombinant human liver isovaleryl-CoA dehydrogenase and the double mutant (Glu254Gly/Ala375Glu were purified as described previously (Mohsen & Vockley, 1995a,b). Glutaryl-CoA dehydrogenase from *Paracoccus denitrificans* was a generous gift of Dr. Mazhar Husain (Byron et al., 1990). CoASH (lithium salt), isovaleryl chloride, 2,6-dichlorophenolindophenol (DCIP), phenazine methosulfate (PMS), glutaryl-CoA, and 3-methyl-2-butenoyl-CoA (β-methylcrotonyl-CoA) were from Sigma. Ferricenium hexafluorophosphate and butyric and hexanoic anhydrides were purchased from Aldrich. HPLC-grade potassium phosphate was from Fisher.

General Methods. Unless otherwise stated, all buffers were 50 mM potassium phosphate, pH 7.6, containing 0.3 mM EDTA. Static absorbance measurements were performed on a Hewlett-Packard 8452A diode array spectrophotometer. Concentrations of enzymes were determined using the following extinction coefficients: 14.2 mM^{-1} cm^{-1} at 446 nm for SCAD (determined by the GuHCl method; Thorpe et al., 1979) and 14.1 mM^{-1} cm^{-1} at 446 for IVAD (Nishina et al., 1995). Concentration and ultrafiltration of small enzyme samples was achieved using microconcentrators (Amicon Corp., M_r 30 000) according to the manufacturer's instructions. Binding and kinetic parameters were determined from spectrophotometric titrations using Enzfitter (Elsevier Biosoft) or Inplot (Graph Pad Software). Short-chain acyl-CoA dehydrogenase activity was determined by using the ferricenium hexafluorophosphate assay (Lehman et al., 1990). The activity of isovaleryl-CoA dehydrogenase and the double mutant were determined by the DCIP/PMS assay system using isovaleryl-CoA (Mohsen & Vockley, 1995a) and hexanoyl-CoA (Thorpe et al., 1979), respectively.

Synthesis and Purification of Acyl-CoA Thioesters. Butyryl-CoA and hexanoyl-CoA were prepared from the corresponding acid anhydrides by the method of Bernert and Sprecher (1977). Isovaleryl-CoA was synthesized from the corresponding acid chloride (Li et al., 1991). 2-Pentynoyl-CoA (Freund et al., 1985) and 3-methyl-3-butenoyl-CoA (Cummings & Thorpe, 1994) were gifts from Drs. P. J. Powell and J. G. Cummings, respectively. All synthesized thioesters were purified by reverse-phase HPLC with a Perkin-Elmer Series 400 liquid chromatograph on a semi-preparative octadecylsilica column (Vydac) using a gradient of methanol and 50 mM phosphate buffer, pH 5.3 (Corkey et al., 1981).

All acyl-CoA thioesters used in this work were quantitated using an extinction coefficient of 16 mM^{-1} cm^{-1} at 260 nm unless otherwise noted (Stadtman, 1957). 2-Pentynoyl-CoA and 3-methyl-2-butenoyl-CoA were quantitated at 260 nm using extinction coefficients of 20 900 M^{-1} cm^{-1} (Freund et al., 1985) and 22 000 M^{-1} cm^{-1} (Stadtman, 1957), respectively. Chart 1 shows the structures of some of the ligands used and discussed in this paper.

Separation of 3-Methyl-3-butenoyl-CoA and 3-Methyl-2-butenoyl-CoA by Reverse-Phase HPLC. 3-Methyl-3-butenoyl-CoA and 3-methyl-2-butenoyl-CoA were separated on an

analytical C18 reverse-phase column (4.6 × 250 mm, Fisher) with a gradient of methanol and 50 mM phosphate buffer, pH 4.0, using the following program: 6 min 10% methanol/90% phosphate buffer; 30 min linear gradient to 40% methanol; 3 min linear gradient to 60% methanol; 3 min 60% methanol; 3 min linear gradient to 10% methanol; 6 min 10% methanol. Chromatograms were developed at a flow rate of 1.0 mL/min and detected at 260 nm. 3-Methyl-3-butenoyl-CoA and 3-methyl-2-butenoyl-CoA eluted after 28 min and 30 min, respectively. The 3-methyl-3-butenoyl-CoA and 3-methyl-2-butenoyl-CoA detected in the isomerization experiments (see later) coeluted with authentic samples on HPLC.

Inactivation of Glutaryl-CoA Dehydrogenase by 2-Pentynoyl-CoA. Glutaryl-CoA dehydrogenase (2 μM in 50 mM phosphate buffer, pH 6.5, containing 25% ethylene glycol, 25 °C) was incubated in the absence and presence of 50 μM 2-pentynoyl-CoA. Aliquots were periodically withdrawn and assayed for enzymatic activity using the DCIP/PMS assay system with glutaryl-CoA as substrate. The enzyme was rapidly ($t_{1/2}$ < 1 min) inactivated to less than 0.1% residual activity in the presence of 2-pentynoyl-CoA. However, in the absence of the unsaturated thioester, there was no significant decrease in the activity of the dehydrogenase.

Construction of the IVAD Model. Visualization and minimization of protein structures were performed with a Silicon Graphics Indigo II workstation running Insight II (MSI 95.0). Minimizations employed the Discover program using the continuous-valence force field (Discover Users Guide, 1995). Sequence and structural alignments used the Homology program with the Dayhoff scoring matrix (Dayhoff et al., 1983; Homology Users Guide, 1995). Ligand and cofactors were constructed and modified using the Ligand Design program. Coordinates of the crystal structure of medium-chain acyl-CoA dehydrogenase (3MDE) and bacterial butyryl-CoA dehydrogenase (1BUC) were obtained from the Brookhaven Protein Data Bank. Prior to minimization, hydrogens were added to each structure (pH 7), bond order was assigned, water molecules were removed from MCAD, and annotations were deleted from column 13 in both files. Minimizations followed a seven-step protocol as described previously (Trievel et al., 1995). Medium-chain acyl-CoA dehydrogenase (MCAD) and bacterial butyryl-CoA dehydrogenase (BBCD) were aligned structurally using monomer B and the IVAD sequence was aligned to the short-chain enzyme. Coordinates were assigned to IVAD largely from the bacterial enzyme; however, in cases where no coordinates from BBCD were available, those from MCAD were used. Insertions unique to IVAD were modeled using loops constructed *de novo* using a constrained minimization algorithm (Homology Users Guide, 1995). The preliminary structure of IVAD was minimized using the splice repair command to remove steric problems introduced during model building. The flavin cofactor and acetoacetyl-CoA thioester were added to the structure using the Ligand Design program and the holoprotein was minimized in a five-step procedure with increasing degrees of conformational freedom during each stage of minimization. Acetoacetyl-CoA was then converted to 2-pentynoyl-CoA, 3-methyl-3-butenoyl-CoA, or 3-methyl-2-butenoyl-CoA using the Ligand Design program and the complex was reminimized as described above. Double mutants were constructed by replacing the relevant amino acids followed by reminimization of the structures.

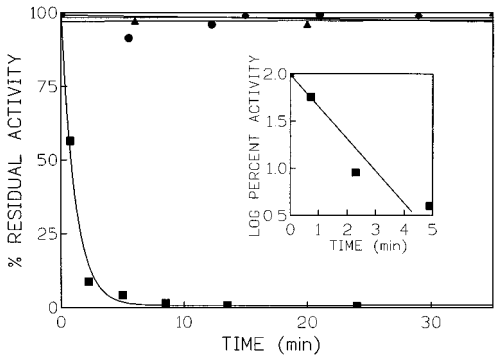


FIGURE 1: Inactivation of short-chain acyl-CoA dehydrogenase and isovaleryl-CoA dehydrogenase by 2-pentynoyl-CoA. Short-chain acyl-CoA dehydrogenase (2 μM in 50 mM phosphate buffer, pH 7.6, 25 °C) was incubated in the absence (●) and presence (■) of 50 μM 2-pentynoyl-CoA. Periodically, aliquots were withdrawn and assayed for activity utilizing the ferricenium assay (see Materials and Methods). The curve represents a first-order decay to 0.4% activity with a rate constant of 0.88 min^{-1}. The inset is a semilogarithmic plot of this data. IVAD (2 μM in 50 mM phosphate buffer, pH 7.6) was also incubated in the presence (▲) and absence (◆) of 50 μM 2-pentynoyl-CoA and assayed as described in Materials and Methods. No significant inactivation of IVAD was observed over 1 h of incubation in the presence of 2-pentynoyl-CoA.

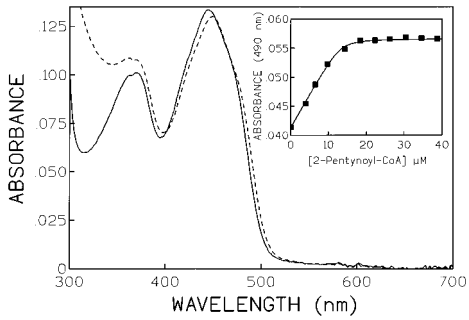


FIGURE 2: Spectrum of isovaleryl-CoA dehydrogenase complexed with 2-pentynoyl-CoA. The spectrum of IVAD (9.5 μM) was determined in the absence (—) and presence (- - -) of 2-pentynoyl-CoA (30 μM) in 50 mM phosphate buffer, pH 7.6. No significant long wavelength band formation was observed over 1 h at 25 °C. The inset plots the increase in absorbance at 490 nm upon titration with 2-pentynoyl-CoA and is fit to a K_d of 1.8 μM.

RESULTS AND DISCUSSION

Interaction between Short-Chain and Isovaleryl-CoA Dehydrogenase and 2-Pentynoyl-CoA. Short-chain acyl-CoA dehydrogenase from pig liver (see Materials and Methods; 2.0 μM in 50 mM phosphate buffer, pH 7.6) was incubated in the presence and absence of 50 μM 2-pentynoyl-CoA at 25 °C. Samples of enzyme were periodically withdrawn and assayed for activity as described in Materials and Methods. In good agreement with earlier studies (Lundberg & Thorpe, 1993), the enzyme was rapidly inactivated to approximately 0.4% residual activity with a first-order rate constant of 0.88 min^{-1} (Figure 1). In contrast, the activity of wild-type isovaleryl-CoA dehydrogenase was unaffected upon incubation with this unsaturated thioester, under the conditions described above (Figure 1, triangles).

Spectral Changes upon Binding. Figure 2 shows the spectral changes induced upon binding of 2-pentynoyl-CoA to isovaleryl-CoA dehydrogenase. Successive additions of ligand give the typical red shift expected for a ligand that binds to the dehydrogenase without subsequent charge

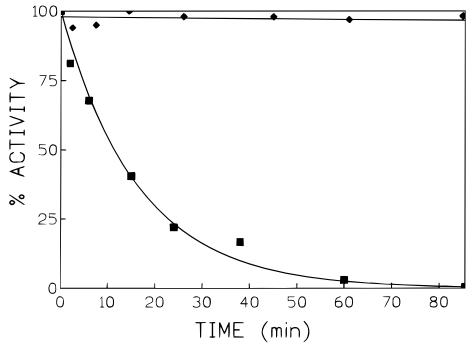


FIGURE 3: Inactivation of isovaleryl-CoA dehydrogenase double mutant by 2-pentynoyl-CoA. IVAD, double mutant (4 μM in 50 mM phosphate buffer, containing 25% ethylene glycol, pH 7.6, 25 °C) was incubated in the absence (◆) and presence (■) of 50 μM 2-pentynoyl-CoA. Aliquots were periodically withdrawn and assayed for activity. The curve represents a first-order decay to approximately 0.6% activity with a rate constant of 0.06 min^{-1}.

transfer interactions or reduction of the flavin prosthetic group (Powell et al., 1987). Given a K_d of 1.8 μM (see inset to Figure 1), greater than 90% of isovaleryl-CoA dehydrogenase would be complexed with 2-pentynoyl-CoA. Consequently, the failure to inactivate the enzyme in Figure 1 cannot simply be due to weak binding of the acetylenic thioester. The spectral changes observed in Figure 2 are very similar to those initially observed upon the addition of 2-octynoyl-CoA to the medium-chain dehydrogenase (Freund et al., 1985; Powell & Thorpe, 1988; Peterson et al., 1995). In that case the bound species gives way to a green-colored enolate to flavin charge transfer complex with eventual irreversible inactivation of the enzyme (Freund et al., 1985; Powell et al., 1988). Clearly these subsequent changes do not occur with isovaleryl-CoA dehydrogenase (Figure 2).

The observation that the short- and medium-chain acyl-CoA dehydrogenases are inactivated by 2-alkynoyl-CoA analogues, whereas isovaleryl-CoA dehydrogenase (this work) and the long-chain dehydrogenase from beef liver (Ankele et al., 1996; Lee et al., 1996) are not, suggests that the position of the catalytic base is critical in conferring sensitivity toward these reagents. Figure 3 corroborates this suggestion: the IVAD double mutant Glu254Gly/Ala375Glu becomes sensitive to inhibition with 2-pentynoyl-CoA. Inactivation is approximately 15-fold slower than that observed with SCAD (Figure 1). This sluggishness is consistent with the decreased catalytic activity of the double mutant (4% and 16% that of the wild type using isovaleryl-CoA and hexanoyl-CoA, respectively; Mohsen & Vockley, 1995a). Strong protection against inactivation of the double mutant is observed with the inclusion of 100 μM hexanoyl-CoA (99% activity remained after 27 min in the presence of 100 μM 2-pentynoyl-CoA compared to 25% in the absence of substrate; data not shown). Activity of the double mutant was not regained after removal of excess inhibitor by ultrafiltration (see Materials and Methods).

3-Methyl-2-butenoyl-CoA and 3-Methyl-3-butenoyl-CoA: Probes for C-4 Proton Abstraction. The isomeric 3-methyl-*trans*-2-octenoyl-CoA and 3-methyleneoctanoyl-CoA form the same reduced flavin adduct with the medium-chain dehydrogenase (Cummings & Thorpe, 1994; Scheme 4, step 3, where $-R = -C_5H_{11}$). Flavin adduct formation with the 3-methylene derivative is rapid (steps 1−3), involving abstraction of the relatively acidic α-proton (step 2). γ-Proton abstraction with the methyl analog is considerably slower (Cummings & Thorpe, 1994) leading to the same adduct via steps 4, 5, and 3 of Scheme 4. Thus, these unsaturated isomeric analogs would be expected to probe the ability of a single active-site base to access both C-2 and C-4 protons. Since isovaleryl-CoA dehydrogenase prefers shorter chains, we used 3-methyl-2-butenoyl-CoA and 3-methyl-3-butenoyl-CoA (Chart 1). As a control, the corresponding changes encountered with the short-chain dehydrogenase were assessed.

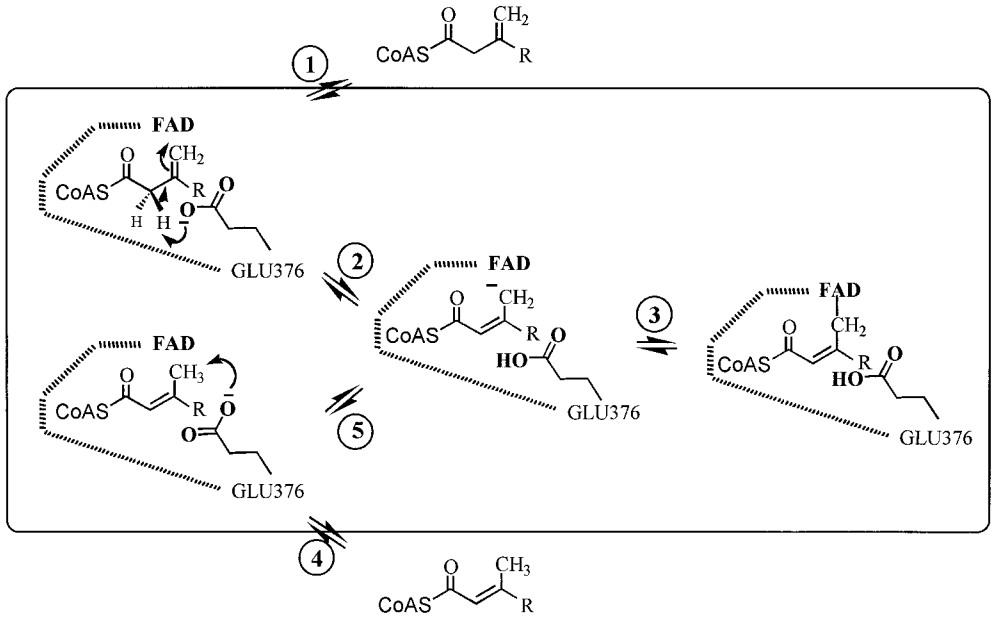
Spectral Changes upon Binding 3-Methyl-3-butenoyl-CoA to the Short-Chain Enzyme. Figure 4 show the spectral changes observed upon the addition of 6 equiv of 3-methyl-3-butenoyl-CoA to the short-chain dehydrogenase. Bleaching of the flavin is rapid (to ca. 35% original absorbance in less than 15 s; curve 2) followed by a partial reappearance of oxidized flavin, which is complete after 20 min (solid symbols, see inset). These changes are analogous to those encountered with the medium-chain dehydrogenase (Cummings & Thorpe, 1994) and reflect the isomerization of the relatively potent, but reversible, 3-methylene inhibitor to the thermodynamically weaker reversible reductant 3-methyl-2-butenoyl-CoA (top to bottom in Scheme 4 via steps 1, 2(3), 5, and 4). This reaction can be conveniently monitored at 260 nm because the double bond is brought into conjugation with the carbonyl group in the 2-enoyl-CoA analogue (not shown; Cummings & Thorpe, 1994). Further, HPLC analysis of supernatants after ultrafiltration showed the expected appearance of 3-methyl-2-butenoyl-CoA after incubation of 3-methyl-3-butenoyl-CoA with the short-chain enzyme (see Materials and Methods).

The addition of 3-methyl-2-butenoyl-CoA to the short-chain dehydrogenase gave relatively slow bleaching of the flavin (k = 1.6 min^{-1}; inset to Figure 4, open circles) to yield exactly the same final spectrum as that observed with the isomeric inhibitor (curve 3). Since activation of the 3-methyl analogue requires γ-proton abstraction, the short-chain dehydrogenase, like the medium-chain enzyme (Cummings & Thorpe, 1994), can abstract protons from either C-2 or C-4 position. In the case of both short- (this work) and medium-chain (Cummings & Thorpe, 1994) dehydrogenases, removal of the less acidic C-4 proton from 3-methyl-2-enoyl-CoA analogs is slower than α-proton abstraction from the 3-methylene derivatives.

Reduction of IVAD with 3-Methyl-3-butenoyl-CoA and Binding of 3-Methyl-2-butenoyl-CoA. Figure 5 shows the spectral changes observed when isovaleryl-CoA dehydrogenase is incubated with 3-methyl-3-butenoyl-CoA. Like the short-chain dehydrogenase, the enzyme was rapidly reduced upon the addition of 3-methyl-3-butenoyl-CoA (via steps 1−3, Scheme 4). However, with isovaleryl-CoA dehydrogenase these spectral changes were stable over 1 h. No significant return of the spectrum of oxidized flavin, which accompanies the isomerization reaction with the short-chain enzyme (Figure 4), is observed with wild-type isovaleryl-CoA dehydrogenase. The absence of isomerase activity was confirmed by HPLC. Supernatants taken from incubation of the enzyme with 3-methyl-3-butenoyl-CoA showed less than 2% isomerization of 200 μM thioester over 2 h using 2 μM wild-type isovaleryl-CoA dehydrogenase.

Since the wild-type isovaleryl-CoA dehydrogenase cannot catalyze this thermodynamically favorable isomerization reaction, reprotonation of the C-4 position must be kinetically

Scheme 4



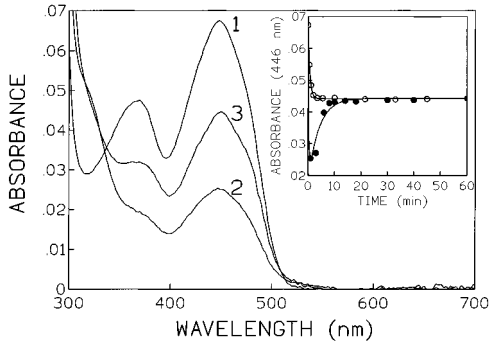


FIGURE 4: Spectrum of short-chain acyl-CoA dehydrogenase in the presence of 3-methyl-3-butenoyl-CoA and 3-methyl-2-butenoyl-CoA. SCAD (5 μM) was mixed with 30 μM 3-methyl-3-butenoyl-CoA in 50 mM phosphate buffer, pH 7.6, 25 °C. Spectra (1−3) were recorded 0.0, 1.0, and 60 min after the addition of the ligand. The inset plots the change in the absorbance at 446 nm over 60 min (●). The experiment was repeated with 30 μM 3-methyl-2-butenoyl-CoA and the absorbance changes are shown in the inset (○).

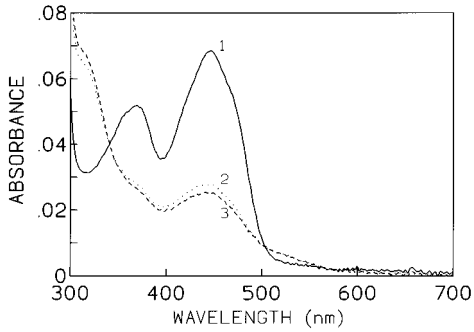


FIGURE 5: Spectrum of isovaleryl-CoA dehydrogenase with 3-methyl-3-butenoyl-CoA. IVAD (5 μM in 50 mM phosphate buffer, pH 7.6) was mixed with 30 μM 3-methyl-3-butenoyl-CoA at 25 °C. Spectra (1−3) were recorded 0, 1, and 60 min after the addition of the acyl thioester. No further changes were observed after an additional 30 μM thioester. The reduced species shown in curve 3 regained full activity when diluted into the standard assay mixture (via steps 3−1, Scheme 4; data not shown; see Materials and Methods).

disfavored. Microscopic reversibility then dictates that 3-methyl-2-butenoyl-CoA should not be a reductant for this enzyme via steps 4, 5, and 3 (Scheme 4). This is the case: 3-methyl-2-butenoyl-CoA causes the expected red shift in the flavin envelope (Powell et al., 1987) without significant reduction of the flavin (data not shown). Subsequent absorbance changes are very minor: over 1 h an approximately 3% reduction of the flavin absorbance at 452 nm was observed (data not shown).

Although the wild-type isovaleryl-CoA dehydrogenase is not an effective isomerase in Scheme 4 (see above), the double mutant shows >90% isomerization to the conjugated thioester under the same conditions (2 μM enzyme, 200 μM 3-methyl-3-butenoyl-CoA, 2 h) as above.

Modeling the Interaction between Thioesters and Wild-Type Isovaleryl-CoA Dehydrogenase and the Double Mutant. The results described above show that short- and medium-chain acyl-CoA dehydrogenases are capable of removing protons from the C-2 or C-4 positions of the unsaturated analogues used here. In addition, preliminary experiments with glutaryl-CoA dehydrogenase, an enzyme with a glutamate base in the corresponding position (Goodman et al., 1992) showed rapid and complete inactivation with 2-pentynoyl-CoA (see Materials and Methods). Glutaryl-CoA dehydrogenase, like short- and medium-chain enzymes, can catalyze an allylic proton shift (as in Scheme 2) and this isomerization proceeds via a monoprotic base with little exchange from solvent protons (Gomes et al., 1981). There can be little doubt that this residue is the catalytic glutamate carried on the loop between helices J and K in these enzymes (Kim et al., 1993; Thorpe & Kim, 1995). Rotation of a carboxylate base could deliver a proton to either α- or γ-position without sizable conformational changes within the active site (Cummings & Thorpe, 1994).

Figures 6 and 7 rationalize why wild-type isovaleryl-CoA dehydrogenase is incapable of rapidly removing a proton at the γ-carbon of either 2-pentynoyl-CoA or 3-methyl-2-butenoyl-CoA. First, molecular modeling of the enzyme substrate complex (see Materials and Methods) shows that Glu254 on the G helix can make van der Waals contact with the *pro-R* α-proton of the normal substrate, isovaleryl-CoA

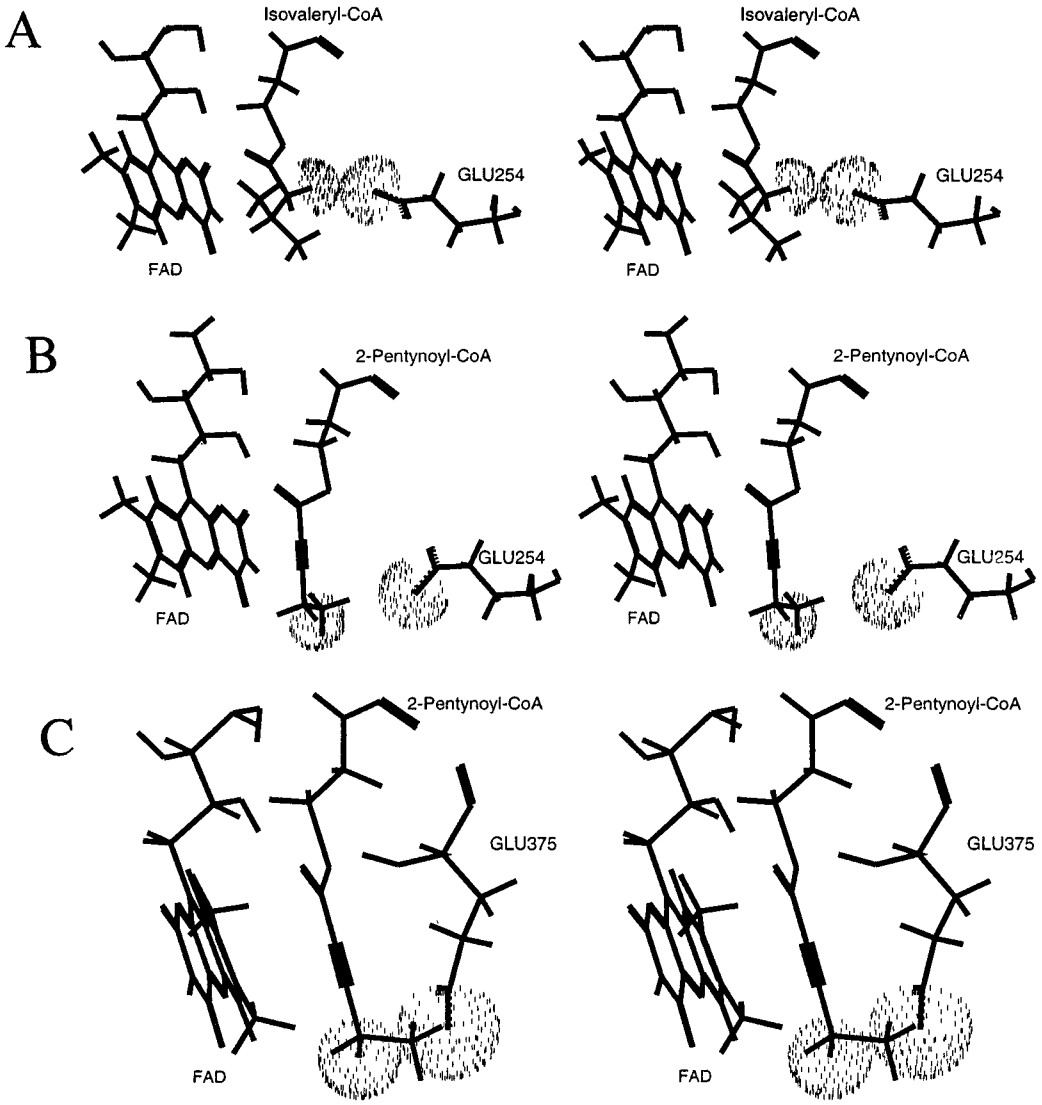


FIGURE 6: Structure of isovaleryl-CoA dehydrogenase complexed with 2-pentynoyl-CoA and isovaleryl-CoA. Stereoviews of the active site of isovaleryl-CoA dehydrogenase complexed with isovaleryl-CoA (panel A) and 2-pentynoyl-CoA (panel B) were constructed and minimized as described in Materials and Methods. Panel C shows the corresponding 2-pentynoyl-CoA complex with the double mutant (Glu254Gly/Ala375Glu). For the purposes of clarity, a portion of the acyl-CoA thioester and the FAD cofactor have been omitted.

(Figure 6, panel A). Abstraction of this proton with elimination of the β-hydrogen as a hydride equivalent leads to reduction of the isoalloxazine ring during normal catalysis. In the corresponding 2-pentynoyl-CoA·enzyme complex (panel B), the carboxylate oxygen of Glu254 is some 4.2 Å from the γ-proton of the bound ligand. Hence 2-pentynoyl-CoA binds to isovaleryl-CoA dehydrogenase as judged by the typical red shift of the flavin envelope (Figure 2) without the subsequent long-wavelength band formation associated with γ-proton abstraction (Freund et al., 1985; Powell et al., 1988; Lundberg & Thorpe, 1993). Panel C shows that migration of the catalytic base to position 375 in the Glu254Gly/Ala375Glu double mutant would bring the γ-proton of 2-pentynoyl-CoA within range of the carboxylate base. This is consistent with the regained sensitivity of the double mutant to 2-pentynoyl-CoA (Figure 3).

Molecular modeling with complexes of wild-type IVAD with 3-methyl-3-butenoyl-CoA and 3-methyl-2-butenoyl-CoA derivatives are further consistent with the arguments developed above. Panel A of Figure 7 depicts the 3-methyl-3-butenoyl-CoA complex, which requires activation by α-proton abstraction (see earlier). The close approach of the *pro-R* α-proton of the bound ligand to the carboxylate oxygen of Glu254 (2.6 Å) is consistent with the rapid bleaching of the flavin chromophore observed in Figure 4. In contrast, the failure of the bound 3-methyl-2-butenoyl-CoA to form the flavin adduct can be explained by the considerably further distance of the γ-proton from the carboxylate (4.1 Å, panel B). Structures of the double mutant allow the base to interact with both α- and γ-positions (panel C), and this is consistent with the ability of the double mutant of isovaleryl-CoA to isomerize 3-methyl-3-butenoyl-CoA to the corresponding 3-methyl-2-butenoyl-CoA analog.

Recently Ghisla and Kim and their colleagues (Nandy et al., 1996b; Lee et al., 1996) have found that the medium-chain acyl-CoA dehydrogenase double mutant Thr255Glu/Glu376Gly is unaffected by 2-octynoyl-CoA. This loss of inhibition toward 2-octynoyl-CoA in the medium-chain enzyme is nicely complementary to the results described in this work, whereby a double mutant of isovaleryl-CoA dehydrogenase becomes sensitive to 2-acetylenic thioesters. However, they now report an apparent exception to the simple correlation that sensitivity toward 2-alkynoyl-CoA analogs is conferred when the carboxylate base is located

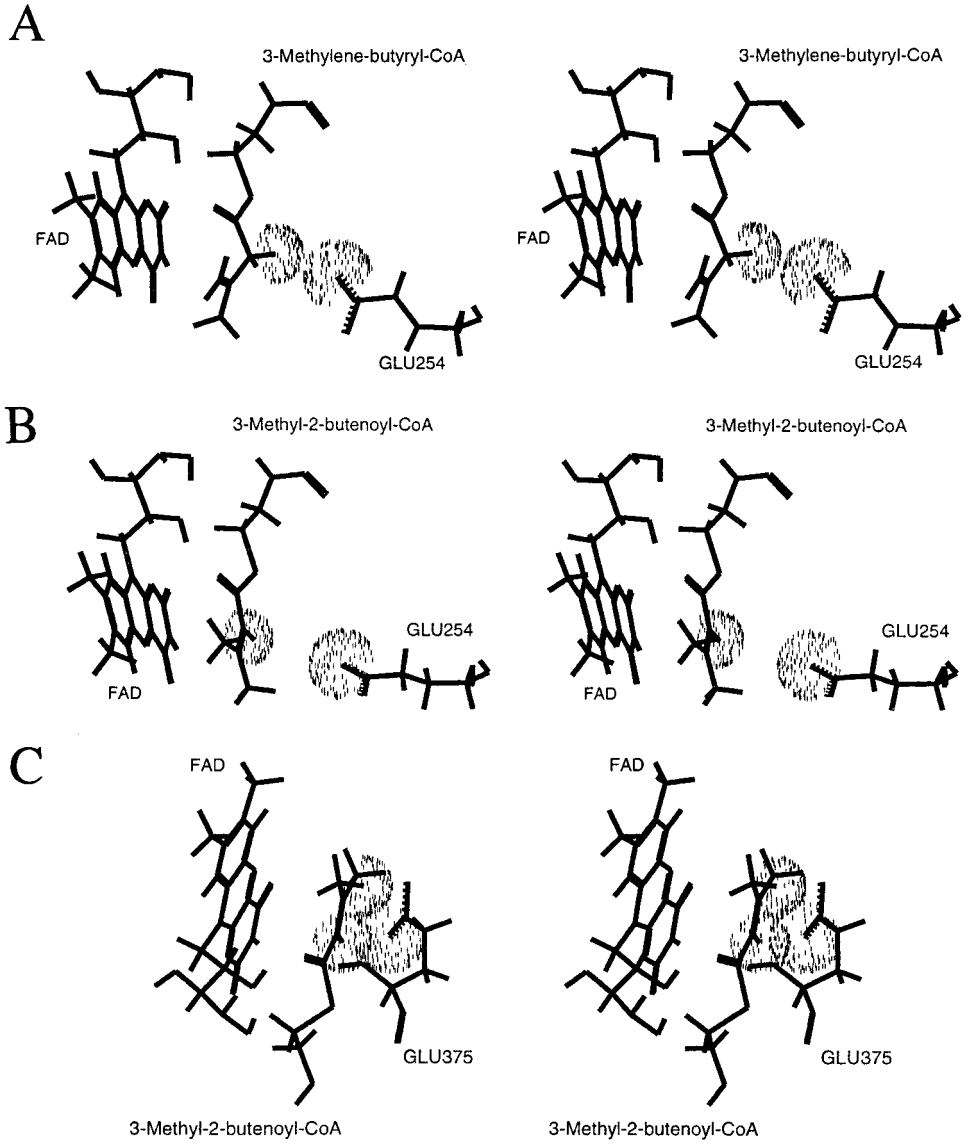


FIGURE 7: Structure of isovaleryl-CoA dehydrogenase complexed with 3-methyl-3-butenoyl-CoA and 3-methyl-2-butenoyl-CoA. The structures of IVAD complexed with 3-methyl-3-butenoyl-CoA (panel A), 3-methyl-2-butenoyl-CoA (panel B), and the double mutant (Glu254Gly/Ala375Glu) modeled with 3-methyl-2-butenoyl-CoA (panel C) were constructed as described in Materials and Methods. Portions of the FAD cofactor and acyl thioester have been deleted for clarity in these stereoviews.

on the loop between J and K helices but not when the base is on the G helix. Thus, although the beef liver long-chain acyl-CoA dehydrogenase fits the correlation and is reported to be resistant to inhibition with 2-octynoyl-CoA (Ankele et al., 1991; Nandy et al., 1996), preliminary data suggest that the human enzyme is sensitive to this inhibitor. The origins of this surprising difference between two long-chain acyl-CoA dehydrogenases are not yet clear.

One issue to be considered with these 2-acetylenic thioesters is that they were not originally envisaged as mechanism-based inactivators but as potential affinity labels (Freund et al., 1985; Powell et al., 1988). Indeed this is presumably why phenylpropynoyl-CoA, a potential Michael acceptor without γ-protons to abstract (Chart 1), is a facile inactivator of the medium-chain dehydrogenase (Freund et al., 1985). Thus inactivation of the acyl-CoA dehydrogenases by 2-alkynoyl-CoA need not necessarily involve γ-proton abstraction, and this might confound the simple correlation described above. However, the availability of a second potential inactivation route does not easily explain why wild-type isovaleryl-CoA dehydrogenase is so refractory toward 2-pentynoyl-CoA. A further issue of importance in interpreting data obtained with 2-alkynoyl-CoA compounds is to ensure that the facile conversion of these unsaturated thioesters to the corresponding 3-keto analogues catalyzed by contaminating traces of enoyl-CoA hydratase (Thorpe, 1986) does not interfere with interpretation of inhibition data.

The studies described here accentuate the key role that the position of the catalytically essential, but mobile, carboxylate base plays in the processing of two different classes of mechanism-based inhibitors of the acyl-CoA dehydrogenase family. Thus the design of selective inhibitors of these enzymes can exploit not only the size and shape of their optimal substrates but also the position of their catalytic bases. In view of the data on the long-chain acyl-CoA dehydrogenase, it will be interesting to characterize their behavior with a range of inhibitors to explore these intriguing differences in behavior.

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