Mechanism-based Inactivation of Human Glutaryl-CoA Dehydrogenase by 2-Pentynoyl-CoA: Rationale for Enhanced Reactivity*

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2-Pentynoyl-CoA inactivates glutaryl-CoA dehydrogenase at a rate that considerably exceeds the rates of inactivation of short chain- and medium chain acyl-CoA dehydrogenases by this inhibitor and related 2-alkynoyl-CoAs. To determine the rate of inactivation by 2-pentynoyl-CoA, we investigated the inactivation in the presence of a non-oxidizable analog, 3-thiaglutaryl-CoA, which competes for the binding site. The enhanced rate of inactivation does not reflect an alteration in specificity for the acyl group, nor does it reflect the covalent modification of a residue other than the active site glutamate. In addition to determining the inactivation of catalytic activity a spectral intermediate was detected by stopped flow spectrophotometry, and the rate constants of formation and decay of this charge transfer complex ($\lambda_{\text{max}} \approx 790$ nm) were determined by global analysis. While the rate-limiting step in the inactivation of the other acyl-CoA dehydrogenases can involve the abstraction of a proton at C-4, this is not the case with glutaryl-CoA dehydrogenase. Glutaryl-CoA dehydrogenase is also differentiated from other acyl-CoA dehydrogenases in that the catalytic base must access both C-2 and C-4 in the normal catalytic pathway. Access to C-4 is not obligatory for the other dehydrogenases. Analysis of the distance from the closest carboxylate oxygen of the glutamate base catalyst to C-4 of a bound acyl-CoA ligand for medium chain-, short chain- and isovaleryl-CoA dehydrogenases suggests that the increased rate of inactivation reflects the carboxylate oxygen to ligand C-4 distance in the binary complexes. This distance for wild type glutaryl-CoA dehydrogenase is not known. Comparison of the rate constants of inactivation and formation of a spectral species between wild type glutaryl-CoA dehydrogenase and a Glu370Asp mutant are consistent with the idea that this
distance in glutaryl-CoA dehydrogenase contributes to the enhanced rate of inactivation and the 1,3-prototropic shift catalyzed by the enzyme.
Several years ago, Schaller and coworkers reported that 2-pentynoyl-CoA rapidly inactivates *Paracoccus denitrificans* glutaryl-CoA dehydrogenase (GCD) \(^1\) with \(t_{1/2}\) less than 1 min \((1)\). We also observed an extremely rapid rate of inactivation of human GCD by 2-pentynoyl-CoA. The oxidative reaction catalyzed by GCD is similar to other acyl-CoA dehydrogenases in that it oxidizes the acyl-CoA to the 2-enoyl-CoA, glutaconyl-CoA \((2)\). However, the catalytic pathway of GCD differs from other members of the acyl-CoA dehydrogenase family because it also catalyzes the decarboxylation of glutaconyl-CoA to crotonyl-CoA. The latter reaction is formally the substitution of a proton at C-4 for CO\(_2\). Glu370 in human GCD abstracts a proton from C-2, prior to hydride transfer from C-3 to the N-5 of FAD, and then functions as a conjugate acid catalyst, Glu370H\(^+\), to transfer the same proton to C-4 of the transient, delocalized crotonyl-CoA anion following decarboxylation of glutaconyl-CoA \((2, 3)\). Several aspects of the mechanism have been investigated with GCD isolated from *Pseudomonas fluorescens* \((3)\). Given the sequence identity between this bacterial GCD and human GCD (64%) and the level of identity and conservative replacements (77%), a common reaction mechanism is likely. Unlike the glutamate base in MCAD and SCAD, Glu370 in human GCD is proposed to access both C-2 and C-4 of the bound acyl-CoA in the normal catalytic pathway \((3)\). Also, mutants of human GCD are available that may also be useful for understanding a possible basis for the extremely rapid rate of inactivation of this dehydrogenase \((4, 5)\).

MCAD and SCAD are both inactivated by 2-alkynoyl-CoAs \((6-8)\). The mechanism of inactivation of these two related dehydrogenases is proposed to involve the initial abstraction of a proton from C-4 of the inhibitor even though it is not mandatory.
that the catalytic base access C-4 during normal catalysis (5). The residues that are covalently modified by these mechanism-based inhibitors, in both MCAD and SCAD, are the glutamate bases that initiate the catalytic pathways by abstracting a C-2 proton prior to hydride transfer to the N-5 of FAD (6, 7).

In the experiments reported here, we investigated the rapid inactivation of GCD by 2-pentynoyl-CoA. The experiments show that the catalytic base, Glu370, is modified in the inactivation process. The rapid inactivation of GCD is, therefore, not due to the covalent modification of another residue. The experiments provide rate constants for the formation and decay of a spectral intermediate in the inactivation pathway along with rate constant for inactivation and equilibrium constants for the binding of 2-pentynoyl-CoA and 3-thiaglutaryl-CoA. Since the rate of inactivation is extremely rapid, the latter constants were determined in the presence of a non-oxidizable analog, 3-thiaglutaryl-CoA, which competes for the active site. A rationale for the enhanced reactivity of GCD based on access of the catalytic base to C-4 of the inhibitor is proposed and is consistent with the rate of inactivation and formation of a charge transfer species in the reaction of 2-pentynoyl-CoA with GCD.
EXPERIMENTAL PROCEDURES

Materials. 2-Pentynoic acid was purchased from GFS. Glutaryl-CoA, acetoacetyl-CoA, CoASH, DTNB, DCPIP, sinapic acid, angiotensin I, and ACTH fragments 1-17, 18-39 and 7-38 were purchased from Sigma. TPCK-trypsin and iodoacetic acid were purchased from Pierce. All other chemicals were of reagent grade. Human wild type and Glu370Asp GCDs were expressed in Escherichia coli, purified and quantitated spectrophotometrically as described before (4). Human ETF was expressed in E. coli and purified as described by Griffin et al. (9).

Enzyme assays. The dehydrogenases were assayed spectrophotometrically (Shimadzu UV-2401 PC spectrophotometer) at 25°C by monitoring the decrease in absorbance at 600 nm. The assays were performed in 50 mM potassium phosphate, pH 7.6, 45 µM glutaryl-CoA, 3 µM ETF and 50 µM DCPIP, the terminal electron acceptor, and started by adding the enzyme (4). Rates were calculated using $\Delta \varepsilon_{600\text{nm}} = 20.1 \text{mM}^{-1}\text{cm}^{-1}$ for the reduction of the dye (4). In these assays, ETF was used in place of 2 mM phenazine methosulfate.

Preparation of acyl-CoAs. 2-Pentynoyl-CoA was synthesized from 2-pentynoic acid by the mixed anhydride method (10), purified as described (11), and quantified using $\varepsilon_{260\text{nm}} = 20.9 \text{mM}^{-1}\text{cm}^{-1}$ (6). 3-Thiaglutaryl-CoA was synthesized as previously described (4). The purity of all synthetic acyl-CoAs was ≥ 98% as determined by analytical high performance liquid chromatography (12).
**Inactivation of GCD.** The reaction of 2-pentynoyl-CoA with GCDs was carried out in 10 mM potassium phosphate, pH 7.0 containing 5 % ethylene glycol at 4°C with an enzyme concentration of 2 µM. All inhibition experiments with 2-pentynoyl-CoA were conducted under pseudo first-order conditions. Samples were withdrawn from the reaction mixtures and assayed at regular intervals to monitor the rate of inactivation (13). In these experiments, it is assumed that the inactivation reaction is terminated upon diluting an aliquot of the reaction mixture into the assay that contains saturating amounts of substrate (13). In the initial experiments, the rate of inactivation was extremely rapid with a t½ of less than 10 s. Alteration of pH and temperature of the inactivation mixture did not slow the inactivation sufficiently. Therefore, further studies were carried out with varying concentrations of 2-pentynoyl-CoA, but in the presence of the non-oxidizable substrate analog, 3-thiaglutaryl-CoA. Addition of this ligand to the reaction mixtures decreased the rate of inactivation by competition of the analog with 2-pentynoyl-CoA at the active site.

The pseudo first-order rate constant of inactivation, $k_{app}$, was calculated using KaleidaGraph 3.5. The data was further analyzed from a plot of $k_{app}$ versus the concentration of 2-pentynoyl-CoA in presence 3-thiaglutaryl-CoA by the "competitive inhibition" model according to Scheme I (14):

![Scheme I](image_url)
where E, I, L, E1, E-I, and E:L refer to enzyme, inhibitor (2-pentynoyl-CoA), ligand (3-thiaglutaryl-CoA), a reversible enzyme-inhibitor complex, an irreversible enzyme-inhibitor covalent complex, and a reversible enzyme-ligand complex, respectively. $K_i$ and $K_L$ are dissociation constants for inhibitor and ligand, respectively, and $k_i$ is the first-order rate constant for the inactivation process. The pseudo first-order rate constant of inactivation, $k_{app}$, is related to $k_i$, $K_i$ and $K_L$ by the following equation:

$$
k_{app} = \frac{k_i}{1 + \frac{K_i}{[I]} \left(1 + \frac{[L]}{K_L}\right)}
$$

These parameters were evaluated by fitting all the data to equation 1, which is of the same form as that describing competitive inhibition (14), by multiple non-linear regression using GraFit 4.0 (15). Each of the data sets was fitted simultaneously to equation 1 with $k_i$, $K_L$ and $K_i$ as the shared parameters.

**Stopped-flow kinetics and data analyses:** The rapid reaction kinetics of 2-pentynoyl-CoA with the dehydrogenases were monitored with an Applied Photophysics SX.18MV-R Stopped-Flow Reaction Analyser equipped with 256 element photodiode array detector and xenon lamp. The dead time of the instrument is about 1.0 ms in this configuration using a cell (20 µl) with a 2.0 mm path length. All concentrations cited refer to final concentrations after mixing. Wild type or Glu370Asp GCD was reacted with 2-pentynoyl-CoA at 4°C or 25°C in 10 mM phosphate buffer, pH 7.0, 5% ethylene glycol. The reaction was monitored over 500 s at 4°C and 100 s at 25°C on a log time scale with spectra accumulated in the 330-1050 nm region at a spectral resolution of about 3.3 nm yielding a data matrix of 500 × 198.
The data were analyzed using Pro-K global analysis/simulation software supplied with the instrument. Global optimization of reaction parameters was achieved by using the Marquardt-Levenberg algorithm and the fitting to models by numerical integration. The data was analyzed simultaneously at all wavelengths by curve fitting to an irreversible sequential first-order model:

\[
\begin{array}{ccc}
A & \rightarrow & B & \rightarrow & C \\
& k_f & & k_s & \\
\end{array}
\]

where \( k_f \) and \( k_s \) are the fast and slow first-order rate constants related to the spectral species, A, B, and C. The absorbance at any given wavelength, \( A_{\lambda} \), is then given by a sum of two exponentials

\[
A_{\lambda} = A_{A\lambda} e^{-kt_f} + A_{B\lambda} e^{-kt_s} + A_{C\lambda}
\]

where the \( k_f \) and \( k_s \), being independent of wavelength, are averaged. The amplitudes, \( A_{A\lambda}, A_{B\lambda}, \) and \( A_{C\lambda} \) allow the calculation of the spectrum and kinetic profiles of the three spectral species, A, B and C. Since interchanging the values of the rate constants \( k_f \) and \( k_s \) leads to non-meaningful calculated spectra, the assignment of fast and slow phases to either \( k_f \) or \( k_s \) becomes unequivocal.

**Mass spectrometry.** The covalent modification of GCD by 2-pentynoyl-CoA was detected by mass spectrometry. Wild type GCD (16.6 \( \mu \)M) was inactivated with a 5-fold excess of 2-pentynoyl-CoA in 10 mM ammonium acetate, pH 7.5 at room temperature for 5 min. As a control wild type enzyme without inhibitor was processed similarly. The molecular mass of a subunit of native and inactivated wild type GCD was determined by
ESI mass spectrometry as described below. The ESI mass spectral experiments were carried out by coupling a protein-trapping column to a Q-Tof2™ mass spectrometer (Micromass Ltd, Manchester, UK). Mass spectra were acquired with the time of flight analyzer at pusher frequency 16129 Hz covering the mass range 1000 to 2500 amu and accumulating data for 5 s per cycle. Time to mass calibration was made with CsI cluster ions acquired under the same conditions. The cone voltage was 60 eV. The collision cell was maintained at 12 eV without gas. Protein samples were desalted by loading protein onto a protein-trapping column (15 × 1 mm) hand packed with a polymeric reverse phase resin (PRP-1, Hamilton Co., Reno, Nevada), with 1% acetic acid at 150 µl/min flow rate. Elution of proteins was carried out with 90% methanol / 0.5% formic acid at a flow rate of 20 µl/min, using a syringe pump (Harvard Apparatus 22). Mass spectrometric data were processed using MassLynx software provided by Micromass Ltd.

Identification of the modified peptide was accomplished by MALDI-TOF mass spectrometry of the trypsin-digested protein. Native and modified wild type GCD with 2-pentynoyl-CoA (as above) were reduced with dithiothreitol and alkylated with iodoacetate in 6 M guanidinium chloride (16). After extensive dialysis against distilled water, the proteins were lyophilized and resuspended in 10 mM NH₄HCO₃. The proteins were then digested overnight with 2% w/w TPCK-trypsin at room temperature (17). The digested samples were analyzed by MALDI-TOF mass spectrometry (Voyager DE-PRO, Perseptive Biosystems, Framingham, MA) in linear and reflector modes using sinapic acid (10 mg/ml in 80:20 v/v mixture of acetonitrile and 0.1% TFA, 0.5 ml) as the MALDI matrix. Spectra were externally calibrated to angiotensin I, and the ACTH
fragments 1-17, 18-39 and 7-38. The amino acid sequence of human GCD precursor protein (18), which includes the 44 amino acid mitochondrial targeting sequence, can be accessed through NCBI protein database under NCBI Accession Number Q92947.

Quantitation of free CoAS anion. The release of CoAS anion from covalently inactivated GCD was quantitated by reaction with DTNB by two methods. The first method was that described by Freund and coworkers (6). In the second method, GCD was inactivated by addition of 10-fold molar excess of 2-pentynoyl-CoA at 4°C for one hour in 300 µl of 10 mM potassium phosphate buffer, pH 7.0, containing 5% ethylene glycol. The enzyme was removed from the reaction mixture by filtering through Centricon centrifugal membrane filter (YM-30) and the free CoAS anion in the filtrate was quantitated using 0.1 mM DTNB as above, again using ε₄₁₂nm = 14.1 mM⁻¹cm⁻¹ (19). The results obtained by the two methods agreed within ± 10%.

Database searches and molecular modeling. The amino acid sequence of the mature GCD (18) was used to search against the Pseudomonas fluorescens genome in the database of the United States Department of Energy Joint Genome Institute using TBLASTN (20). The Pseudomonas GCD was located in Contig302 using the following parameters: percent identity, 40% and minimum matching length, 20.

The distances between the nearest carboxylate oxygen of the glutamate catalytic base to the C-4 of the substrate in the crystal structures of MCAD (PDB: 3MDE) (21), SCAD (PDB: 1JQI) (22), and IVD (PDB: 1IVH) (1, 23) were determined using Insight II 97.0 package of modeling software on a Silicon Graphics Indigo 2 workstation as
described (1, 24). MCAD and SCAD were co-crystallized with ligands, octanoyl-CoA and acetoacetyl-CoA, respectively (21, 22), whereas isovaleryl-CoA was modeled into the active site of wild type IVD as described (1, 24).
RESULTS

Kinetics of inactivation of GCD by 2-pentynoyl-CoA. The inactivation of human wild type GCD by 2-pentynoyl-CoA was extremely rapid with a t½ that was less than 10 s at 4°C. To characterize the inactivation quantitatively, the apparent rate of inactivation was determined at several different concentrations of a second ligand that binds to the active site. The ligand chosen was 3-thiaglutaryl-CoA, a redox inactive analog of glutaryl-CoA (3). Figure 1 shows a plot of \( k_{\text{app}} \) versus the concentration of inhibitor in presence of protection afforded by the substrate analog, 3-thiaglutaryl-CoA. The analysis of the 20 experimental data points when fitted to the competitive inhibition model converged in 5 iterations with a reduced \( \chi^2 = 2.6 \times 10^{-6} \). The maximum value for the first-order rate constant of inactivation, \( k_i \) (extrapolated to zero concentration of 3-thiaglutaryl-CoA and infinite inhibitor concentration) is 4.3 \( \pm \) 0.4 min\(^{-1}\). This rate constant is too rapid to be measured in the absence of protection by a competing ligand. The apparent dissociation constants for the binding of 2-pentynoyl-CoA, \( K_i \), and 3-thiaglutaryl-CoA, \( K_L \), were 6.9 \( \mu \)M and 22.8 \( \mu \)M, respectively. This indicates that the inhibitor, 2-pentynoyl-CoA binds tightly, and the value for 3-thiaglutaryl-CoA agrees with our earlier determinations (4, 5).

We also compared the pseudo first-order rate constants for the inactivation of wild type GCD and Glu370Asp GCD (Fig. 2). The reactions were conducted at 4°C in incubations containing 135 \( \mu \)M 3-thiaglutaryl-CoA, 20 \( \mu \)M 2-pentynoyl-CoA and 2 \( \mu \)M dehydrogenase in 10 mM potassium phosphate, pH 7.0, 5% ethylene glycol. The values of \( k_{\text{app}} \) for inactivation of wild type and Glu370Asp GCD are 1.16 \( \pm \) 0.07 and 0.26 \( \pm \) 0.01 min\(^{-1}\), respectively. The 4.5-fold lower rate constant of inactivation of the mutant may be explained by the difference in distance (~1Å) from the \( \gamma \)-carboxylate of Glu370 to the C-
4 proton (or C-2) compared with the β-carboxylate of Asp370. Alternatively, the difference in rate constants may also be explained by the distance from the Michael donor, carboxylate, to the Michael acceptor, 2,3-pentadienoyl-CoA, which is an intermediate formed in the inactivation process (6). It is also possible that there is a difference in the orientation of the carboxylate as it functions as a catalytic base, conjugate acid or nucleophile in the Michael addition (25).

Release of CoAS⁻ anion and stoichiometry of modification. As is the case with MCAD (6), the CoAS⁻ anion is liberated upon covalent modification of wild type GCD and Glu370Asp GCD by 2-pentynoyl-CoA. The amount of CoAS⁻ anion released from the inactivated wild type GCD was 0.98 ± 0.10 per site as determined by reaction with DTNB as described in Experimental Procedures. The stoichiometry of CoAS⁻ anion released from inactivated Glu370Asp GCD was 1.12 ± 0.11 per site.

Evidence for covalent modification of the protein was obtained by mass spectrometry of the unmodified wild type and modified enzymes. The average mass of the unmodified subunit determined by ESI mass spectrometry was 43601.2 Da; the average mass of the subunit based on the primary sequence deduced from the DNA coding sequence in the expression vector is 43598.0 Da (18). Dissociation of the tetrameric enzyme to subunits and loss of FAD occurs during the processing of the sample (see Experimental Procedures). The average mass of the inactivated subunit determined by ESI mass spectrometry is 43699.1 Da (data not shown); an increase of 97.9 Da, corresponding to C₅H₆O₂, which is consistent with the mass of the expected adduct following release of CoAS⁻ anion.
Identification of Glu370 as the site of chemical modification. Table 1 shows the observed masses of the tryptic peptides from native GCD along with their amino acid sequence. The numbering of the peptides reported here is for mature human GCD without the 44 amino acid mitochondrial targeting sequence, but includes the amino terminal methionine required for expression from the vector in the case of peptide 1-25 (see footnotes, Table 1). Comparison of tryptic maps of the native and 2-pentynoyl-CoA modified enzyme by MALDI-TOF mass spectrometry showed an additional peptide (m/z = 2775.2) that is 98.6 mass units greater than the peptide (m/z = 2676.6) containing residues 359-382 (Fig. 3, Table 1). The average mass predicted for the latter peptide, [M + H]+ is 2677.0. The peptide 359-382 has five residues that could act as a catalytic base in the enzymatic reaction: His359, Glu364, Glu370, His373, Asp374, His376, and Arg382. Of these, only Glu370 is positioned appropriately to function as the catalytic base (26). Further, Glu370 in human GCD occupies the same position as Glu376 in MCAD and Glu368 in SCAD (18, 27). Finally, substitution of Glu370 by glutamine drastically reduces the catalytic activity of GCD to 0.04% of wild type and substitution by aspartate reduces activity 14-fold (4) and rate constant of inactivation 4.5-fold. Some native peptide was also detected in the analysis of peptides from the inactivated protein although the reaction was run to completion as judged by the complete loss of catalytic activity and absence of significant native monomer as determined by ESI mass spectrometry (Fig. 3B). The covalent derivative of the glutamate in porcine short-chain acyl-CoA dehydrogenase is known to be alkali-labile, as are the acyl-CoA dehydrogenases modified by 3-alkynoyl-CoAs (3, 28). Some adduct is likely lost during reductive alkylation and trypsin digestion which
are conducted at pH 8. Incubation of the peptides generated from unmodified and modified GCD at room temperature for 16 h in 0.1 M NH₄OH resulted in the complete conversion of the modified peptide to the unmodified peptide but no change in the profile of the other peptides (Fig. 3C). This result is consistent with the contention that Glu370 is the modified residue and that Glu370 reacts with the allene intermediate in a Michael addition forming an ester linkage.

*Spectral changes and rapid kinetics of interaction of GCD with 2-pentynoyl-CoA.* Fig. 4A shows stopped-flow spectral data in which 460 µM 2-pentynoyl-CoA was mixed with 54 µM wild-type GCD at 4°C in 10 mM potassium phosphate, pH 7.0, 5% ethylene glycol. The flavin absorbance at 447 nm decreases and the long wavelength absorption (λₘₐₓ ≈ 791 nm) is immediately observed on mixing; the latter absorbance decays over a period of about 500 s.

Singular value decomposition (SVD) analysis of the spectral kinetic data provided at least three significant singular values. A minimal description of the data indicates a two-step model with three spectral species (A, B, and C). Global analyses of these data according to Scheme II yielded the two pseudo first-order rate constants, \( k_f = 61.98 \pm 0.11 \text{ min}^{-1} \) and \( k_s = 0.94 \pm 0.01 \text{ min}^{-1} \). The convergence occurred in five iterations with a variance of \( 5.126 \times 10^{-4} \). Similarly, data obtained at 25°C, when analyzed as above, yielded the two first-order rate constants, \( k_f = 231.3 \pm 1.07 \text{ min}^{-1} \) and \( k_s = 4.15 \pm 0.01 \text{ min}^{-1} \) showing increased values of \( k_f \) and \( k_s \) by factors of 3.73 and 4.42, respectively, compared to the data at 4°C (data at 25°C not shown).
Rate constants determined by single wavelength analysis (791 nm) are identical to those obtained by global analysis (Fig. 4B). The spectral species, B, with absorbance at 791 nm reaches a maximum of 96% and an apparent steady state in about 2 s (Fig. 4B). This apparent steady state persists between 2 and 6 s (Fig. 4B). The calculated spectrum of species B (Fig. 5A) indicates that it is the spectral intermediate that exhibits the charge transfer band at long wavelength. Formation of species C occurs only after about 6 s (Fig. 5B). The value of $\varepsilon_{791\text{nm}}$ is 2.1 mM$^{-1}$ cm$^{-1}$ which is comparable to the charge transfer species exhibited by MCAD and SCAD during inactivation by 2-pentynoyl-CoA (6, 8).

The results are consistent with the formation of a delocalized acyl-CoA anion ($\lambda_{\text{max}} \approx 791$ nm) that forms a charge transfer complex with the electron-deficient flavin (29). This charge transfer species may be either the delocalized C-4 anion generated by deprotonation or the proposed enolate (29) that forms after attack of the catalytic glutamate on the intermediate allene (30). Both anions can be stabilized by hydrogen bonding of the enolate oxygen to the 2'-hydroxyl of FAD and the backbone NH of Glu370 (26). The enolate subsequently decays with the release of CoAS$^-$ anion from the covalently modified protein (6, 8). The charge transfer species that accompanies inactivation in GCD forms much faster than the corresponding species of MCAD or SCAD (6, 8).

When the mutant dehydrogenase, Glu370Asp (37µM) was mixed with 560 µM 2-pentynoyl-CoA at 4°C there was no increase in long wavelength absorbance (Fig. 6). Nonetheless, the mutant is irreversibly inactivated and the final spectrum is similar to that of the wild type dehydrogenase following inactivation. SVD analysis indicated three
spectral species. Thus, global analysis according to Scheme II, yields two pseudo first-order rate constants, $k_f = 14.72 \pm 0.05 \text{ min}^{-1}$ and $k_s = 0.19 \pm 0.01 \text{ min}^{-1}$ (Fig. 6A). The convergence occurred in five iterations with a variance of $4.209 \times 10^{-4}$. These rate constants are 4.2- and 3.3-fold slower than those determined with the wild type GCD, and comparable to the difference in rate constants of inactivation of wild type and Glu370Asp GCDs. The calculated spectra of the individual species yielded $\lambda_{\text{max}} = 446 \text{ nm}$ and $\epsilon_{446\text{nm}} = 14.7 \text{ mM}^{-1} \text{ cm}^{-1}$ for A, $\lambda_{\text{max}} = 456 \text{ nm}$ for B ($\epsilon = 14.3 \text{ mM}^{-1} \text{ cm}^{-1}$) and C ($\epsilon = 12.4 \text{ mM}^{-1} \text{ cm}^{-1}$) (Fig 6B). The concentration of B reaches a maximum of 95% and remains in this apparent steady state level between 15 and 21 s. Formation of the spectral species, C, occurs after 21 s, and does not reach completion during the 500 s duration of this experiment. The absence of a long wavelength intermediate is similar to the observation when MCAD is inactivated with either propiolyl-CoA, phenylpropiolyl-CoA or 2-octynoyl-pantetheine and points out the steric requirement for charge transfer complex formation (6).
DISCUSSION

Mechanism-based inhibitors have been useful tools for the investigation of acyl-CoA dehydrogenase catalysis, and in one case, permitted discrimination among otherwise very closely related members of the acyl-CoA dehydrogenase family based on a single structural difference related to function (1). Like *P. denitrificans* GCD (1), human GCD is rapidly inactivated by 2-pentynoyl-CoA with $t_{1/2}$ less than 10 sec at 4°C. The rate constant of inactivation greatly exceeds those of MCAD and SCAD by 2-pentynoyl-CoA and 2-octynoyl-CoA at 25°C (6-8). Significantly, 2-pentynoyl-CoA does not inactivate IVD (1). The location and function of Glu370 in GCD are identical to the homologous catalytic glutamate residue located on the loop between the J and K helices in MCAD and SCAD (22). The catalytic glutamate is positioned on the G helix in IVD but has the same function as those in GCD, MCAD and SCAD (22).

The mechanism of inactivation of MCAD and SCAD by 2-alkynoyl-CoAs involves initial abstraction of a proton at C-4 followed by isomerization of the delocalized C-4 anion to 2,3-pentadienoyl-CoA and covalent modification of the glutamate catalytic base by a Michael addition (7, 8). It is likely that the pK$_a$ of the C-4 protons of 2-pentynoyl-CoA is comparable to that of a C-2 proton of the natural acyl-CoA substrates. Proton transfer from C-2 to the base catalyst of GCD initiates the catalytic pathway of GCD, as is the case with MCAD and SCAD (31). However, this protonated glutamate of GCD is also thought to function as a conjugate acid catalyst to protonate the crotonyl-CoA anion following decarboxylation of the enzyme-bound intermediate, glutaconyl-CoA (8, 9). The proposed mechanism for inactivation of acyl-
CoA dehydrogenases by 2-alkynoyl-CoAs is shown in Scheme III.

\[ E + I \xrightarrow{k_1} E\cdot I_1 \xrightarrow{k_2} E\cdot I_2^* \xrightarrow{k_3} E\cdot I_3 \xrightarrow{k_4} E\cdot I_4^* \]

Scheme III

Inactivation involves a Michaelis complex, EI₁, between the enzyme and inhibitor, a delocalized C-4 anion charge transfer complex with the enzyme, EI₂⁺, and a non-covalent complex between 2,3-pentadienoyl-CoA and the enzyme, EI₃. Attack of Glu370 on the 2,3-diene yields a covalent complex between a delocalized enolate of the acyl-CoA. This enolate is expected to participate in a second charge transfer complex, EI₄⁺, with the oxidized flavin. EI₄⁺ subsequently hydrolyzes to free CoA and the five carbon carboxylic acid covalently bound to the catalytic glutamate through an ester bond, EI₅.

With the exception of the charge transfer complex that absorbs at 790 nm, which could be either EI₂⁺ or EI₄⁺, multiple intermediates could not be identified in this investigation or in any previous work. However, it is not unreasonable that the rapid formation of the charge transfer complex reflects the formation of the C-4 anion. The decay of this long wavelength-absorbing species reflects the decay of the covalent enolate that involves hydrolysis of the thioester. The bracketed regions in Scheme III indicating \( k_f \) and \( k_s \) are the observed first-order rate constants referring to the formation and decay of spectral
species B in Scheme II that is detected by stopped-flow experiments. The individual reactions in Scheme III cannot be resolved since there are few spectral changes associated with the different chemical steps. Also, binding of 2-pentynoyl-CoA is rapid and could not be observed. The first spectral species in the global analysis, A, is the Michaelis complex. The spectral species with the charge transfer band, B, reflects both C-4 anion and the enolate. The bound 2,3-pentadienoyl-CoA is not expected to form a charge transfer complex since it is electron deficient. The scheme proposes two anionic forms forming charge transfer band that are separated by a non-absorbing species at long wavelength. Yet a decrease in absorbance at 790 nm is not detected suggesting that the intermediate allene reacts rapidly with Glu370 and the two charge transfer complexes cannot be resolved. The decay of the charge transfer spectral intermediate presumably occurs upon substitution of a water molecule for the liberated CoA. The final spectral species, C, is the covalently inactivated enzyme. Therefore, the only rate constants that can be resolved are for the formation of the delocalized C-4 anion \( k_f = 61.98 \text{ min}^{-1} \) and the decay of the enolate due to hydrolysis of the thioester bond and release of CoAS\(^-\) \( k_s = 0.94 \text{ min}^{-1} \).

Formation of the apparent C-4 anion is rate-limiting in the inactivation of MCAD by 2-octynoyl-CoA because formation of this charge transfer complex and the rate constant of inactivation both show a primary deuterium isotope effect of 6-7 when MCAD is reacted with \((4,4-d_2)\) 2-octynoyl-CoA \((6)\). The ratio, \( k_f/k_i \), is 2.3 for this reaction. The corresponding ratio for GCD is 15. Assuming that formation of the C-4 anion by GCD is also reflected by formation of the charge transfer species \( (\lambda_{\text{max}} \approx 790 \text{ nm}) \), formation of the C-4 anion is not rate limiting in the inactivation of GCD.
The results presented here are consistent with the modification of Glu370 in the active site of human GCD. Therefore, the high rate of inactivation of GCD relative to that observed with other acyl-CoA dehydrogenases is not the result of covalent modification of a residue other than the glutamate base catalyst. Also, it is unlikely that the large difference in the rate constant of inactivation results from a difference among acyl group specificities of the three dehydrogenases. The enzymes have different chain length specificities and GCD exhibits specificity for the carboxylate substituent at C-4 (4, 11). Differences among the active site cavities of MCAD, SCAD, IVD and GCD have not been extensively compared (32); however, the active sites of both GCD and SCAD are shallower than that of MCAD and the cavity of GCD is different in that a cationic residue, Arg94, is present at the bottom of the binding pocket (21, 22, 26, 32). The shallow cavity may influence the capacity of the enzyme to polarize the inhibitor (29). That polarization could influence the pKa of hydrogens at C-4, or render C-3 of the resulting 2,3-dieneoyl-CoA a better acceptor in the Michael reaction with Glu370 (29). This is also unlikely to be the dominant factor because the rate constants of inactivation and formation of the long wavelength intermediate of GCD considerably exceed the rate constants found with SCAD (8). Also, $k_i$ for inactivation of MCAD by 2-pentynoyl-CoA and 2-octynoyl-CoA are identical (6).

Abeles and coworkers proposed the catalytic mechanism of GCD in their investigations of *Pseudomonas fluorescencs* GCD (3). Their work indicated that a general base catalyzes a 1,3-prototropic shift because no significant amount of tritium from solvent is incorporated into the crotonyl-pantetheine product (3). The sequence conservation between human and *Pseudomonas* GCDs includes residues corresponding
to Glu370 and Arg94 of human GCD. In human GCD, Arg94 is within hydrogen bonding distance of the \( \gamma \)-carboxylate of glutaryl-CoA and functions as an electrostatic catalyst (5, 26). Other conserved residues in the active site of the bacterial enzyme correspond to Ser98, Val99, Leu103, Phe133, Leu246 and Tyr369 of the human enzyme (26). Given the sequence identity between the bacterial GCD and human GCD (64%) and the level of identity and conservative replacements (77%), a common reaction mechanism is almost certain. Therefore, Glu370 of human GCD is expected to access both C-2 and C-4 of the bound acyl-CoA in the catalytic cycle (3). This access is not essential for catalysis by the other dehydrogenases. Thus, there may be structural differences between MCAD, SCAD, IVD and GCD. These differences permit Glu370 in GCD to reach both C-4 and C-2 of glutaryl-CoA and crotonyl-CoA more easily to ensure an efficient 1,3-prototropic shift. This is reflected in the rapid inactivation of GCD by 2-pentynoyl-CoA. The relative rate constants of inactivation of wild type MCAD and GCD by 2-octynoyl-CoA and 2-pentynoyl-CoA, respectively, can be compared with the rate constants of inactivation of the corresponding mutants in which the glutamate base catalysts have been replaced by aspartate residues. The Glu370Asp mutant of human GCD is inactivated about 4-fold more slowly than the wild type, while the Glu376Asp mutant of MCAD is inactivated 16-fold more slowly (33). These data are consistent with the idea that the carboxylate base of GCD has easier access to C-4 of the 2-alkynoic-CoA than the carboxylate base of MCAD.

Schaller et al. showed that IVD is not irreversibly inactivated by 2-pentynoyl-CoA unless the catalytic base is moved from its normal position on the G helix to the loop between the J and K helices (1). Those investigators proposed that wild type IVD
probably failed to react because the active site base could not reach the proton at C-4 of 2-pentynoyl-CoA (1). GCD represents the other extreme of the reactivity spectrum from IVD. Table 2 summarizes the rate constants of inactivation, $k_i$, and the spectral rate constants, $k_f$ and $k_s$, for the formation and decay of the long wavelength-absorbing intermediates of MCAD, SCAD, IVD and GCD in the reactions with 2-pentynoyl-CoA, or 2-octynoyl-CoA in the case of MCAD. The distances from the nearest carboxylate oxygen of the catalytic base glutamate to C-4 of the bound ligand were determined from the crystal structures or molecular modeling. In these structures, the ligands are octanoyl-CoA in the case of porcine MCAD and acetoacetyl-CoA in the case of rat SCAD (21, 22). Isovaleryl-CoA was modeled into the structure of IVD. The data indicate an inverse relationship between the distance from the carboxylate oxygen to ligand C-4 and the rate constant of inactivation by a 2-alkynoyl-CoA. We do not have this distance for GCD; however, the 4-fold difference in $k_{app}$ for inactivation of wild type GCD versus Glu370Asp GCD indicates that the same general relationship between distance, $k_i$ and $k_f$ holds for GCD. These data suggest that the distance from the carboxylate oxygen of Glu370 GCD to C-4 is less than that in SCAD, i.e., less than 3.5 Å. This rationale does not consider the most favorable angle of approach to the hydrogen by the oxygen.

Examples of other enzymes that catalyze proton shifts are triose phosphate isomerase and 3-oxo-$\Delta^5$-steroid isomerase (34, 35). Glu165 in triose phosphate isomerase functions as a catalytic base to abstract a C-1 proton from the substrate in the Michaelis complex (34). The distance between the carboxylate oxygen of Glu165 and C-1 and C-2 of the substrate, 3.0 Å, is shorter than the distance normally associated with C-
H⋯O hydrogen bonds and optimal for proton transfer between C-1 and the carboxylate oxygen (34). The typical distance for C⋯O hydrogen bonds is 3.5 Å, though shorter distances, between 2.0 and 3.0 Å, are observed in other cases (36, 37), as in the case of the Glu165 carboxylate oxygen and C1 of dihydroxyacetone phosphate (34). An oxygen atom approaching a carbon atom along the C-H bond is not expected to be closer than 3.7 Å unless there is a cohesive interaction (36,37). These cohesive interactions are also strongly dependent on the stereochemistry as expected for hydrogen bonds (36, 38).

Asp38 of 3-oxo-Δ5-steroid isomerase catalyzes a 1,3-prototropic shift (35). Asp38 is located 2.8 Å above the 4Cβ proton (35). The carboxylate residues of both isomerases also exhibit considerable conformational flexibility (34, 35).

The reactivity of GCD with 2-pentynoyl-CoA may reflect a similarly short distance between the carboxylate oxygen of Glu370 and C-4 of the inhibitor, as well as some conformational mobility of Glu370. The data presented here provide support for the proposed 1,3-prototropic shift catalyzed by the general base catalyst in GCD and a structural basis for that efficient proton shift. We also suggest that the greatly enhanced rate of inactivation of GCD by 2-pentynoyl-CoA compared with other members of the acyl-CoA dehydrogenase family results from structural differences that reflect the efficiency of the proton shift, a unique aspect of the GCD mechanism among the acyl-CoA dehydrogenases.
**Acknowledgments.** We thank Dr. Todd D. Williams and Ms. Homigol Biesiada of the University of Kansas Mass Spectrometry Laboratory at Lawrence for acquiring ESI mass spectra. We also thank Dr. Kim Fung and Dr. Mark Duncan, Biochemical Mass Spectrometry Facility, School of Pharmacy, Univ. of Colorado Health Sciences Center, Denver for acquiring MALDI-TOF mass spectra.
REFERENCES


FOOTNOTES

1 The abbreviations used are: ACTH, adrenocorticotropic hormone; DCPIP, 2,6-dichlorophenol indophenol; DTNB, 5,5-dithiobis (2-nitrobenzoic acid); ESI, electrospray ionization; ETF, electron transferring flavoprotein; FAD, flavin adenine dinucleotide; GCD, glutaryl-coenzyme A dehydrogenase; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; MCAD, medium-chain acyl-CoA dehydrogenase; SCAD, short-chain acyl-CoA dehydrogenase; IVD, isovaleryl-CoA dehydrogenase; SVD, singular value decomposition.
FIGURE LEGENDS

Figure 1. Rates of inactivation of human GCD as a function of 2-pentynoyl-CoA in the presence of varying concentrations of 3-thiaglutaryl-CoA. The concentrations of 3-thiaglutaryl-CoA in the inactivation reactions were 90, 135, 200, and 266 µM. The data were analyzed according to the competitive inhibition model using GraFit 4.0. The smooth curves are the best fit to the experimental data yielding the parameters: $k_i = 4.3$ min$^{-1}$, $K_i = 6.9$ µM and $K_L = 22.8$ µM.

Figure 2. Comparison of the rates of inhibition of wild type GCD and Glu370Asp GCD by 2-pentynoyl-CoA in the presence of 3-thiaglutaryl-CoA. The rates of inactivation of wild type GCD (•) and Glu370Gln GCD (■) were determined in reaction mixtures containing 2 µM enzyme, 20 µM 2-pentynoyl-CoA and 135 µM 3-thiaglutaryl-CoA at 4°C in 10 mM potassium phosphate buffer, pH 7.0 containing 5 % ethylene glycol. The slopes were used to determine $k_{app}$ for each enzyme species.

Figure 3. MALDI-TOF mass spectra of tryptic peptides of native and 2-pentynoyl-CoA inactivated glutaryl-CoA dehydrogenase. The native enzyme (A) and enzyme inactivated with 2-pentynoyl-CoA were reduced and alkylated, digested with trypsin and analyzed by mass spectrometry. The peptides were then treated with 0.1 M ammonium hydroxide, 16 h, 25°C, and the peptides were analyzed. Panel C shows the relevant region of the mass spectrum of the modified enzyme treated with base. The native peptide, residues 359-382 with m/z of 2676.63, shows a 98.6 Da increase after modification and loses this mass, 98.6 Da, upon treatment with ammonium hydroxide. Other peptides observed are
reported in Table 1.

Figure 4. Rapid kinetic analysis of the reaction of 2-pentynoyl-CoA with wild type GCD. In panel A, wild type enzyme (54 µM) and 2-pentynoyl-CoA (460 µM) were mixed at 4°C in the stopped flow spectrophotometer with a photodiode array detector. The time of accumulation of the selected spectra are: (1) 0.0032 s; (2) 0.5613 s; (3) 4.9950 s; (4) 20.780 s; (5) 140.70 s; and (6) 475.90 s. The reaction was carried out in 10 mM phosphate buffer, pH 7.0, 5% ethylene glycol. Global analyses of the data according to Scheme II, a minimal step mechanism, yielded $k_f = 61.98 \pm 0.11 \text{ min}^{-1}$ and $k_s = 0.94 \pm 0.01 \text{ min}^{-1}$. In panel B, the data were analyzed at 791 nm as a function of time. The data at this single wavelength were fit to the equation describing an irreversible sequential first-order model, $A \rightarrow B \rightarrow C$. The analysis yield $k_f = 60.86 \pm 0.96 \text{ min}^{-1}$ and $k_s = 0.64 \pm 0.01 \text{ min}^{-1}$. The inset to Panel B shows the same data over the first 6 s to show the formation of the charge transfer species. Note that the absorbance remains at its maximum value during 2 to 6 s period.

Figure 5. Changes in the concentration of the individual species as a function of time during the reaction of wild type GCD with 2-pentynoyl-CoA. These analyses are based on the experiment shown in Fig. 4A. Panel A, shows calculated spectra of the individual species obtained by global analyses. Panel B shows the time dependence of the concentrations of the three spectral species, a, b, and c, defined in Scheme II and corresponding to the spectra in Panel A. The inset of Panel B shows data over the first 6 s of the reaction.
Figure 6. Rapid kinetic analysis of the reaction of 2-pentynoyl-CoA with Glu370Asp GCD. In panel A, 37 μM enzyme and 560 μM 2-pentynoyl-CoA were mixed at 4°C in the stopped flow spectrophotometer with a photodiode array detector. The time of accumulation of the selected spectra are: (1) 0.0032 s; (2) 0.5613s; (3) 4.9950 s; (4) 20.780 s; (5) 140.70 s; and (6) 475.90 s. The reaction was carried out in 10 mM phosphate buffer, pH 7.0, 5% ethylene glycol. Global analyses of the data according to Scheme II yielded $k_f = 14.72 \pm 0.05 \text{ min}^{-1}$ and $k_s = 0.19 \pm 0.01 \text{ min}^{-1}$. Panel B shows calculated spectra of the three species a, b, and c from global analyses of data in panel A. The inset to panel B shows changes in the concentration of the individual species as a function of time.
Table 1: Tryptic map of native, mature human glutaryl-CoA dehydrogenase in the mass range 2000 – 4000 Da

<table>
<thead>
<tr>
<th>Observed</th>
<th>Residue Number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass (m/z)</td>
<td>Amino Acid Sequence</td>
<td></td>
</tr>
<tr>
<td>2008.4</td>
<td>TWITNSPMADLFVVWAR</td>
<td>167-183</td>
</tr>
<tr>
<td>2110.5</td>
<td>YGIAWGVLGASEFCLHTAR</td>
<td>251-269</td>
</tr>
<tr>
<td>2227.4</td>
<td>LADMLTEITLGLHACLQLGR</td>
<td>292-311</td>
</tr>
<tr>
<td>2571.5</td>
<td>SAMSVQSSLVMHPIYAYGSEEQR</td>
<td>95-117</td>
</tr>
<tr>
<td>2676.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HAMNLEAVNTYEHTDIHALILGR</td>
<td>359-382</td>
</tr>
<tr>
<td>3088.1</td>
<td>MRPEFDWQDPLVLEEQLTDEILIR</td>
<td>1-25</td>
</tr>
<tr>
<td>3733.4</td>
<td>ASATGMIIMDGVEVPEENVLPGASSLGGPGFGLNNAR</td>
<td>214-250</td>
</tr>
</tbody>
</table>

<sup>a</sup>The numbering of the residues refer to the mature human sequence except peptide 1-25, which includes a methionine residue required for expression.

<sup>b</sup>The modified peptide has an additional peak at m/z 2775.2, which up on treatment with ammonium hydroxide reverts back to m/z 2676.8 (also see Figure 3).
Table 2. The relation between the carboxylate oxygen to ligand C-4 distance and the rate constants of inactivation by 2-alkynoyl-CoAs, the formation ($k_f$) and the decay ($k_s$) of the long wavelength absorbing intermediate(s) during the inactivation reaction.

<table>
<thead>
<tr>
<th>Enzyme, Conditions</th>
<th>$k_f$ (min$^{-1}$)</th>
<th>$k_s$ (min$^{-1}$)</th>
<th>$k_i$ (min$^{-1}$)</th>
<th>distance O--C$_4$ (Å)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCD, 4°C</td>
<td>61.98</td>
<td>0.94</td>
<td>4.3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GCD, 25°C</td>
<td>231.3</td>
<td>4.15</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>GCD, E370D, 4°C</td>
<td>14.72</td>
<td>0.19</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>SCAD, 25°C</td>
<td>6.0</td>
<td>2.0</td>
<td>0.9</td>
<td>3.5</td>
<td>(22)</td>
</tr>
<tr>
<td><em>a</em>MCAD, 25°C</td>
<td>1.1</td>
<td>0.06</td>
<td>0.6</td>
<td>4.9</td>
<td>(21)</td>
</tr>
<tr>
<td><em>b</em>MCAD, 25°C</td>
<td>1.4</td>
<td>0.05</td>
<td>0.5</td>
<td>4.9</td>
<td>(21)</td>
</tr>
<tr>
<td>IVD, 25°C</td>
<td>no inactivation</td>
<td></td>
<td></td>
<td>6.3</td>
<td>(23)</td>
</tr>
</tbody>
</table>

*a* Inactivation by 2-octynoyl-CoA (21)

*b* Inactivation by 2-pentynoyl-CoA (21)
Figure 1

[Graph showing the relationship between $k_{app}$ (S$^{-1}$) and [2-Pentynoyl-CoA] (µM). The graph includes curves and data points for different concentrations of 90, 135, 200, and 260 µM, plotted against the [2-Pentynoyl-CoA] concentration.]
Figure 2
Figure 3 A, B and C
Figure 4 A and B
Figure 5 A and B
Figure 6 A and B