Protonation of Crotonyl-CoA Dienolate by Human Glutaryl-CoA Dehydrogenase Occurs by Solvent-Derived Protons†

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ABSTRACT: The protonation of crotonyl-CoA dienolate following decarboxylation of glutaconyl-CoA by glutaryl-CoA dehydrogenase was investigated. Although it is generally held that the active sites of acyl-CoA dehydrogenases are desolvated when substrate binds, recent evidence has established that water has access to the active site in these binary complexes of glutaryl-CoA dehydrogenase. The present investigation shows that the dehydrogenase catalyzes (a) a rapid exchange of C-4 methyl protons of crotonyl-CoA with bulk solvent and (b) protonation of crotonyl-CoA dienolate by solvent-derived protons under single turnover conditions. Both of the reactions require the catalytic base, Glu370. These findings indicate that decarboxylation proceeds via a dienolate intermediate. The involvement of water in catalysis by glutaryl-CoA dehydrogenase was previously unrecognized and is in conflict with a classically held intramolecular 1,3-prototropic shift for protonation of crotonyl-CoA dienolate.

Glutaryl-CoA dehydrogenase (GCD)[1] catalyzes the oxidative decarboxylation of glutaryl-CoA to form crotonyl-CoA and CO_2 and transfers electrons to the respiratory chain via electron transfer flavoprotein (*1*). The catalytic mechanism of the reductive half-reaction of the enzyme with glutaryl-CoA appears to be essentially identical to that of medium-chain acyl-CoA dehydrogenase (*2*, *3*). The reaction mechanism of medium-chain acyl-CoA dehydrogenase has been investigated in detail. The sequence of the chemical steps in the catalytic mechanism is (i) the abstraction of an α-proton of the acyl-CoA substrate by the catalytic base followed by (ii) hydride transfer from the C-3 position of the substrate to N(5) of the FAD (*3*). For GCD, the similarity of mechanism is supported by (a) crystallographic studies of the binary complex between GCD and the alternate substrate, 4-nitrobutyryl-CoA, (b) site-directed mutagenesis of the catalytic base, Glu370, and (c) covalent modification of Glu370 by the active site directed inhibitor, 2-pentynoyl-CoA (*2*, *4*, *5*). GCD also catalyzes the decarboxylation of glutaconyl-CoA, the enzyme-bound product of the reductive half-reaction (*1*, *6*). Formally, decarboxylation is the substitution of a carboxylate group by a proton. In the case of GCD, protonation at the C-4 position, following decarboxylation, could occur (i) directly by solvent, (ii) by solvent via an amino acid side chain, or (iii) by direct intramolecular proton transfer from C-2 to C-4 via the catalytic base, Glu370. [Glu370 refers to the carboxylate form of the residue whereas Glu370(H^+) refers to the conjugate acid]. Such an intramolecular 1,3-prototropic shift, proposed by Gomes and co-workers (*7*), suggests that the active site is shielded from solvent and that water plays no part in catalysis. Intramolecular 1,3-prototropic shift was suggested by Gomes and co-workers because they found that the *Pseudomonas* enzyme did not catalyze exchange of C-2 hydrogens in the absence of turnover and that solvent protons were not incorporated into C-4 of crotonyl-CoA during turnover (*7*). However, recent results from this laboratory are not consistent with an active site that is shielded from solvent. First, GCD can catalyze the hydration of crotonyl-CoA to give 3-hydroxybutyryl-CoA, indicating that water must have access to the active site (*6*). Second, the cleavage of the $CoAS^-$ anion upon covalent modification of Glu370 in GCD by 2-pentynoyl-CoA indicates access of water to the region of the thioester carbonyl (*5*).

In the experiments described here, we show that human GCD catalyzes the rapid exchange of C-4 hydrogens of crotonyl-CoA with solvent protons. Further, single turnover experiments are consistent with the contention that the C-2 hydrogen abstracted from glutaryl-CoA by Glu370 exchanges very rapidly with solvent protons which then protonates a transient crotonyl-CoA dienolate (*6*) following the decarboxylation.

EXPERIMENTAL PROCEDURES

Materials. Coenzyme A, *proteo*-glutaryl-CoA, *proteo*-crotonyl-CoA, and *proteo*-octanoyl-CoA were obtained from

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[1] Abbreviations: GCD, glutaryl-CoA dehydrogenase; $FcPF_6$, ferrocenium hexafluorophosphate.

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Sigma. *2,2,4,4-d*$_4$-Glutaric acid (98% D) was purchased from Cambridge Isotopes Laboratories. Phenyl 2-chloroacetate was purchased from Maybridge (Cornwall, England), and standardized solutions of sodium hydroxide and hydrochloric acid were from Fisher Scientific. Deuterium oxide (99.9% D), trifluoroacetic anhydride, and ferrocenium hexafluorophosphate ($FcPF_6$) were purchased from Aldrich.

Deuterated Buffer. D_2O buffer was prepared by lyophilizing a known volume of 50 mM potassium phosphate buffer, pH 7.6, followed by dissolving the buffer salts in a small volume of D_2O and lyophilizing. Finally, the buffer salts were dissolved in the same known volume of D_2O and stored in an airtight container. When stored for extended periods, D_2O buffer was frozen at −20 °C.

Purification of Glutaryl-CoA Dehydrogenase. Human wild-type and Glu370Gln GCD was expressed in *Escherichia coli*, purified, quantitated, and assayed as described earlier (*2*).

Exchange of Enzyme into Deuterated Buffer. Purified GCD was passed through a desalting column (Amersham/Pharmacia HiTrap desalting 5 mL) to exchange the enzyme from the storage buffer into 50 mM potassium phosphate, pH 7.6, on AKTA FPLC, concentrated to a small volume with a Centricon YM-30 centrifugal filter, and diluted with D_2O buffer. The deuteration procedure of concentrating and diluting in D_2O buffer was performed at least twice. Activity and the spectrum of the enzyme were determined before use.

Synthesis of 2,2,4,4-d$_4$*-Glutaryl-CoA*. *2,2,4,4-d*$_4$-Glutaryl-CoA was synthesized from the anhydride (*8*), purified by DEAE-cellulose anion-exchange chromatography, and desalted on Sephadex G-10. The labeled glutaric anhydride was prepared by refluxing *2,2,4,4-d*$_4$-glutaric acid with trifluoroacetic anhydride and recrystallizing from diethyl ether (mp = 56 °C; lit. mp 55−57 °C). The purity of *2,2,4,4-d*$_4$-glutaryl-CoA (>96%) was determined by analytical HPLC (*9*). The ^{1}H NMR spectrum was consistent with the expected structure. The mass spectrum showed >95% of *2,2,4,4-d*$_4$-glutaryl-CoA [$C_{26}H_{38}(^2H)_4N_7O_{19}P_3S$; exact mass 885.2] and about 5% of d_3-glutaryl-CoA (see Figure 5).

Characterization of the Crotonyl-CoA by NMR. Three experiments were performed: (1) *proteo*-Crotonyl-CoA (1 mM) was incubated anaerobically with wild-type GCD (1 μM) in the presence and absence of $FcPF_6$ (2 mM) in D_2O buffer for 30 min at 25 °C (no turnover). (2) Crotonyl-CoA was generated from *proteo*-glutaryl-CoA (1 mM), $FcPF_6$ (2 mM), and wild-type GCD (1 μM) in D_2O buffer at 25 °C for 30 min (catalytic multiple turnovers); turnover was monitored at 300 nm due to the reduction of $FcPF_6$ using a 0.1 cm path length cuvette. (3) Crotonyl-CoA was also generated by incubation of *2,2,4,4-d*$_4$-glutaryl-CoA (1 mM) with $FcPF_6$ (2 mM) and wild-type GCD (1 μM) in potassium phosphate buffer, pH 7.6, for 30 min at 25 °C [identical to conditions of turnover in (2) but with deuterated substrate in aqueous buffer]. Reactions were quenched by adjusting the pH to 5.0 with 0.5 M phosphoric acid. The enzyme and precipitated electron acceptor were removed by centrifugal filtration using a Centriplus YM-30 (Amicon). Crotonyl-CoA from each reaction was purified by semipreparative HPLC (*9*), desalted on Sephadex G-10, and analyzed by 500 MHz ^{1}H NMR and/or ^{2}H NMR (equivalent to 76.7 MHz) in D_2O.

Kinetics of Enzyme-Catalyzed Solvent Exchange of proteo-Crotonyl-CoA and proteo-Glutaryl-CoA. A known amount of *proteo*-crotonyl-CoA or *proteo*-glutaryl-CoA was lyophilized to give 1.0 mM in 650 μL of D_2O buffer. The 500 MHz ^{1}H NMR spectrum of *proteo*-crotonyl-CoA or *proteo*-glutaryl-CoA was recorded before addition of enzyme. Wild-type or Glu370Gln GCD, 0.6 μM, in D_2O buffer was added to this at 25 °C, and proton spectra were collected as a function of time. The initial spectrum was recorded some 292 s after addition of the enzyme, and eight scans were averaged at every 41.3 s interval thereafter. The integrated intensity of 4^c methyl protons of crotonyl-CoA at 1.75 ppm and 2^c and 3^c methylene protons of glutaryl-CoA at 2.20 and 1.86 ppm, respectively, is in comparison to the pantetheine 6″-methylene resonance at 2.44 ppm as an internal standard (*10*). The first-order rate constant, k_{obs}, was calculated according to the equation:

$$F_t = (F_0 - F_\infty)e^{-k_{obs}t} + F_\infty \quad (1)$$

where F_0, F_t, and F_∞ are the number of protons remaining at zero time, time t, and infinite time, respectively, and k_{obs} is the apparent exchange rate constant. The first-order rate constant for exchange, k_{exch}, was calculated from the equation (*11*):

$$k_{exch} = \frac{n[L]k_{obs}}{[E]} \quad (2)$$

where n is the number of exchangeable protons and [L] and [E] are concentrations of either *proteo*-crotonyl-CoA or *proteo*-glutaryl-CoA ligands and enzyme sites, respectively. Under these conditions, based on K_i for crotonyl-CoA (*6*) or K_m of substrate, the active site is saturated with ligand at all times.

Characterization of the Enzymatically Generated Crotonyl-CoA by Mass Spectrometry. *proteo*-Glutaryl-CoA (1 mM) was converted to crotonyl-CoA by incubation with 1 μM GCD and 2 mM $FcPF_6$ for 30 min in D_2O buffer at 25 °C. Crotonyl-CoA generated during extensive turnover of GCD was isolated as described above for NMR analysis and analyzed by electrospray liquid chromatography mass spectrometry (ESI-LCMS) using a hybrid quadrupole-time-of-flight tandem mass spectrometer (Micromass Q-Tof2, Milford, MA). The mass spectrometer was operated in the positive ion and Tof scan modes. Samples were introduced into the mass spectrometer with a fast LC gradient of 20−94% solvent B over 10 min via a 1 mm reversed-phase C18 column. Solvent A consisted of 0.08% formic acid and 1% methanol in water, and solvent B was 90% acetonitrile and 0.06% formic acid in water containing 25 mM ammonium acetate. Crotonyl-CoA eluted in this system with a retention time of 1.96 min.

Single Turnover of GCD with 2,2,4,4-d$_4$*-Glutaryl-CoA and FcPF*$_6$. Single turnover kinetic analysis was conducted using a KinTek RQF-3 quench flow apparatus (*12*). GCD, 82 μM, in one syringe was rapidly mixed with 0.2 mM *2,2,4,4-d*$_4$-glutaryl-CoA and 1.5 mM $FcPF_6$ in a second syringe. The reactants were equilibrated at 25 °C prior to mixing. The reaction was rapidly quenched by adjusting the pH to 3.4 with 95 mM H_3PO_4 from a third syringe at the times indicated. The concentrations of enzyme and substrate were chosen to yield >90% of active sites bound by substrate (*13*). An excess of the electron acceptor, $FcPF_6$, was used to drive the reaction to completion, thus allowing the reaction time

to be the only variable to satisfy single turnover conditions. After quenching, the denatured enzyme was removed with a Microcon YM-50 centrifugal filter (Millipore) and the filtrate analyzed by liquid chromatography−mass spectrometry (LC-ESI-MS) as described below. As an internal reference standard, 40 μM *proteo*-octanoyl-CoA was added to the filtrate. The KinTek RQF-3 quench-flow apparatus was calibrated using the base-catalyzed hydrolysis of phenyl 2-chloroacetate with standardized HCl as quencher (*14*). This reaction is preferred for calibration since it has a half-life of about 2.7 ms in 1 M NaOH ($k = 363 \pm 21$ M^{-1} s^{-1} at 20 °C), which is close to the dead time of the apparatus, and is conveniently monitored spectrophotometrically using an extinction coefficient of 1450 M^{-1} cm^{-1} for phenol (in water), in contrast to the base-catalyzed hydrolysis of 2,4-dinitrophenyl acetate with a second-order rate constant of 49.4 M^{-1} s^{-1} at 25 °C (*15*).

Quantitative Analysis of Single Turnover Kinetics by LC-ESI−Mass Spectrometry. The concentrations of the substrate, *2,2,4,4-d*$_4$-glutaryl-CoA, and the major product, *d*$_3$-crotonyl-CoA, were determined by selective ion monitoring (SIM) of their respective $[M - H^+]^-$ ions since greater sensitivity and less background signal were observed in the negative ion mode. Negative ion electrospray ionization (ESI) mass spectra were obtained with a PE Sciex (Foster City, CA) API-3000 triple quadrupole mass spectrometer with a turbo ion spray source interfaced to a PE Sciex 200 HPLC system with an Alltech (Deerfield, IL) C-8 column, 7.5 × 4.6 mm. The LC solvent was programmed from 100% 10 mM ammonium acetate, pH 9.0 (1 min hold), to 90% acetonitrile containing 10 mM ammonium acetate in 2 min with an additional 2 min hold at a flow rate of 300 μL/min and sample injection volume of 20 μL. The retention times of *2,2,4,4-d*$_4$-glutaryl-CoA, *d*$_3$-crotonyl-CoA and *proteo*-octanoyl-CoA (added internal reference standard) were 1.8, 2.1, and 2.2 min, respectively.

The mass spectrometer settings were as follows turbo ion spray temperature, 250 °C; needle spray voltage, −3500 V; declustering potential (DP), −30 V; focus plate (FP), −120 V. The reaction of *2,2,4,4-d*$_4$-glutaryl-CoA with glutaryl-CoA dehydrogenase was quantified in SIM mode by measuring the ratio of the respective $[M - H^+]^-$ ion abundances at *m*/*z* 884.2 (*2,2,4,4-d*$_4$-glutaryl-CoA) and *m*/*z* 837.2 (*d*$_3$-crotonyl-CoA) to *m*/*z* 892.2 (*proteo*-octanoyl-CoA, the internal standard) with an integration time of 350 ms per ion per cycle. Standard curves ($R^2 > 0.99$) were determined using varying concentrations of standard solutions of *2,2,4,4-d*$_4$-glutaryl-CoA (0−70 μM) and *proteo*-crotonyl-CoA (0−50 μM) with a fixed concentration of *proteo*-octanoyl-CoA (40 μM).

RESULTS

Incubation of Crotonyl-CoA with GCD or Generation of Crotonyl-CoA by Multiple Turnovers. Based on the work of Gomes and co-workers (*7*), with *2,2,4,4-d*$_4$-glutaryl-CoA as substrate and the reaction run to completion, C-4 of crotonyl-CoA would contain only deuterium. Similarly, if the reaction were run with *proteo*-glutaryl-CoA in D_2O buffer, the methyl group of crotonyl-CoA would contain only hydrogens. However, recent experiments in this laboratory suggest that solvent water has access to the active site of GCD (*5*, *6*),

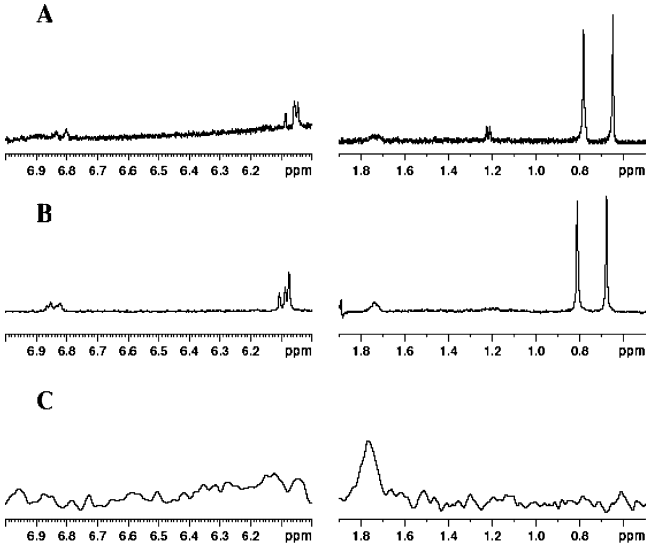


FIGURE 1: NMR spectra of crotonyl-CoA over the region 0.6−2.0 and 6.0−7.0 ppm. Spectra were recorded in D_2O at 25 °C. The methyl groups of crotonyl-CoA are labeled 10″ ($\delta = 0.84$ ppm), 11″ ($\delta = 0.71$ ppm), and 4^c ($\delta = 1.75$ ppm) while 2^c ($\delta = 6.08$ ppm) and 3^c ($\delta = 6.80$ ppm) represent hydrogens across the *trans* double bond. The doublet at 6.05 ppm actually belongs to the 1′ methylene of the ribose moiety of coenzyme A. The numbering system of crotonyl-CoA is according to Wu and co-workers (*16*). Panel A: ^{1}H NMR spectrum (500 MHz) of *proteo*-crotonyl-CoA incubated for 30 min at 25 °C with wild-type GCD (no turnover) in D_2O buffer. Panel B: ^{1}H NMR spectrum (500 MHz) of crotonyl-CoA prepared enzymatically from *proteo*-glutaryl-CoA and $FcPF_6$ for 30 min at 25 °C in D_2O buffer. Panel C: ^{2}H NMR spectrum (76.7 MHz) of the sample in panel B. ^{1}H NMR of *proteo*-crotonyl-CoA, as control, shows δ 0.84 (s, 3H, H10″), 0.71 (s, 3H, H11″), 1.75 (dd, 3H, 4^c), 6.08 (m, 1H, 2^c), and 6.80 (dq, 1H, 3^c), which agrees with assignments in the literature (data not shown).

which contradicts X-ray crystallographic data suggesting that binding of substrate strips water molecules completely from the acyl region of the active site in acyl-CoA dehydrogenases (*4*). In our initial experiments, incubations were conducted at 25 °C for 30 min with $FcPF_6$ as the electron acceptor; crotonyl-CoA was purified (>90% yield) and analyzed by ^{1}H NMR and mass spectrometry. In the first experiment, shown in panel A of Figure 1, incubation of *proteo*-crotonyl-CoA with oxidized enzyme (no turnover) in D_2O buffer resulted in ∼67% exchange of hydrogens of the 4^c methyl group (δ ∼1.75 ppm) of crotonyl-CoA with solvent deuterons as measured by ^{1}H NMR. Identical results were observed in the presence of the electron acceptor. The numbering system of crotonyl-CoA is according to Wu and co-workers (*16*). In panel B of Figure 1, ∼77% of hydrogens of the 4^c methyl group of crotonyl-CoA exchanged with solvent deuterons, measured by ^{1}H NMR, when crotonyl-CoA was generated enzymatically in D_2O buffer from *proteo*-glutaryl-CoA and $FcPF_6$. Panel C is the same experiment as that shown in panel B but monitored by ^{2}H NMR (76.7 MHz); thus, no signals for 10″ and 11″ methyl groups of the CoA moiety are observed. The ^{1}H NMR spectrum of *proteo*-crotonyl-CoA (control) in D_2O buffer was in agreement with that previously published (*16*) and did not show exchange (data not shown). The broadened signal at ∼1.75 ppm is due to superposition of crotonyl-CoA species containing various isotopomers at the 4^c methyl group such as CD_3, CD_2H, CDH_2, and CH_3. Each of these isotopomers is expected to have a slightly different chemical shift because of the upfield heavy atom

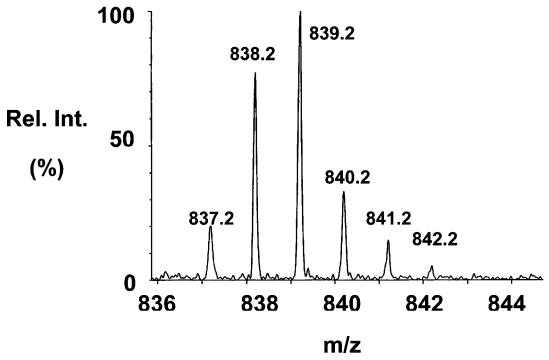


FIGURE 2: Raw data of the positive ion mass spectrum of crotonyl-CoA generated in the presence of GCD (1 μM), $FcPF_6$ (2 mM), and *proteo*-glutaryl-CoA (1 mM) in D_2O buffer over 30 min at 25 °C. The individual molecular species seen are d_1-, d_2-, and d_3-crotonyl-CoA whereas *proteo*-crotonyl-CoA is not observed.

shift that the deuterium exerts on the remaining protons. It is likely that the isotopomers are generated sequentially due to independent rebinding events of crotonyl-CoA in the active site of GCD. For example, statistical distribution of abundance of isotopomers formed due to exchange of methyl hydrogens in acetyldithio-CoA catalyzed by citrate synthase has provided evidence that each exchange process occurs in an independent binding event (*11*). The *trans* hydrogens at the 2^c and 3^c positions (6.08 and 6.80 ppm, respectively) of crotonyl-CoA are nonexchangeable, but the multiplet splitting patterns due to *J* coupling with 4^c methyl protons are removed by exchange of 4^c protons with deuterons. The lines appear significantly narrower owing to very small coupling to 4^c protons.

Crotonyl-CoA generated during extensive turnover of GCD, as above, was analyzed by positive ion LC-ESI-MS in a tandem quadrupole-time-of-flight mass spectrometer (Figure 2). The substrate, 1 mM *proteo*-glutaryl-CoA, was converted to crotonyl-CoA by incubating it with 1 μM GCD and 2 mM $FcPF_6$ for 30 min in D_2O buffer at 25 °C. The molecular species observed have $[M + H^+]^+$ of 837.2, 838.2, and 839.2, representing d_1-, d_2-, and d_3-crotonyl-CoA (Figure 2). These data were corrected for the natural abundance contributions of other isotopes besides deuterium to all of the peaks using the Isotope Pattern Calculator Version 4.0 developed by Junhua Yan (http://www-personal.umich.edu/~junhuay/pattern1.htm). Quantitative analysis suggests that the most abundant species is the *dideutero*-crotonyl-CoA produced by two exchange events (relative abundance of 100%). Similarly, *trideutero*- and *monodeutero*-crotonyl-CoA are observed at 96% and 29% relative abundance, respectively. However, the specific sites of deuteration may not be assigned on the basis of this experiment though the main site of exchange would be the 4^c methyl of crotonyl-CoA. The higher mass isotopic distributions ($m/z > 840.2$) are typical of organic compounds containing C, H, N, O, P, and S. The mass distribution below *m*/*z* 840.2 relates to various isotopomers observed in NMR experiments (Figure 1). Of importance is the fact that the exchange of the hydrogens on the 4^c methyl group of crotonyl-CoA with solvent hydrogen is catalyzed by GCD, whether crotonyl-CoA is added directly or generated by enzyme turnover.

If the 1,3-prototropic shift suggested by Gomes and coworkers (*7*) were operative, one would expect the product

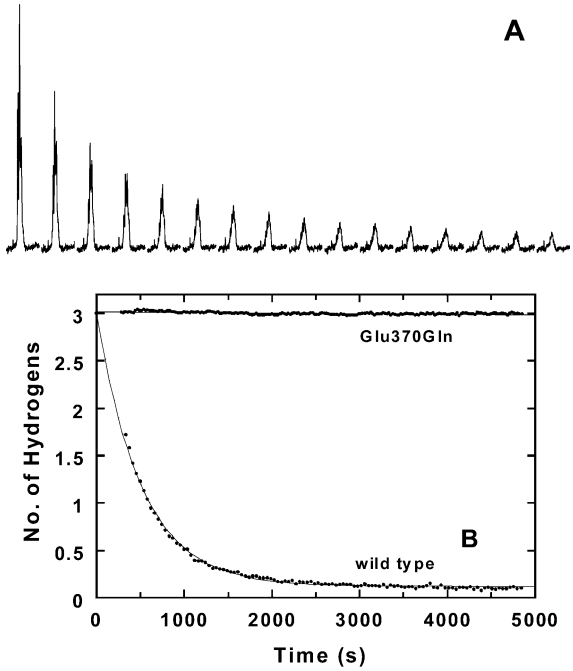


FIGURE 3: Kinetics of proton exchange at the 4^c methyl position of *proteo*-crotonyl-CoA catalyzed by wild-type and Glu370Gln glutaryl-CoA dehydrogenase. (A) Time dependence of the methyl proton resonance during incubation of 1.0 mM *proteo*-crotonyl-CoA with 0.6 μM wild-type GCD in D_2O buffer at 25 °C. Data were accumulated at 41.3 s intervals, and the initial spectrum was recorded 333 s after addition of wild-type enzyme; every fifth spectrum over 1 h is shown. (B) Pseudo-first-order decay of the integrated number of methyl protons with wild-type GCD, derived from the data in panel A, and the integrated number of methyl protons with Glu370Gln GCD. The integrated intensity of the 4^c methyl protons at 1.75 ppm is in comparison to the pantetheine 6″-methylene resonance at 2.44 ppm as an internal standard. The integral of three for the zero time point is an extrapolated value.

generated from *proteo*-glutaryl-CoA in D_2O buffer to be *proteo*-crotonyl-CoA exclusively [*m*/*z* 836.2, $[M + H^+]^+$; CH_3—CH═CH—C(═O)—SCoA; $C_{25}H_{40}N_7O_{17}P_3S$; exact mass 835.2]. Note that *proteo*-crotonyl-CoA is not observed (Figure 2). The extensive enzyme-catalyzed exchange shown in Figures 1 and 2 likely occurs due to rebinding of crotonyl-CoA in the active site of GCD as the concentration of glutaryl-CoA decreases over the course of the reaction. Therefore, it is not clear if the mechanism involves a 1,3-prototropic shift, but this can be evaluated further by using single turnover conditions to preclude rebinding. In any case, the data demonstrate access of solvent-derived protons to the C-4 position of crotonyl-CoA in the active site.

Hydrogen Exchange at C-4 of Crotonyl-CoA with Solvent. We first investigated the possibility that the enzyme catalyzes the exchange of methyl (C-4) protons of crotonyl-CoA by determining the rate of proton exchange. Figure 3A shows the ^1H NMR spectrum of the methyl hydrogens as a function of time when 1.0 mM crotonyl-CoA was incubated with 0.6 μM GCD in D_2O buffer at 25 °C. Figure 3B shows the decrease in the C-4 ^1H NMR signal as a function of time. The pseudo-first-order rate constant, k_{obs}, is $(200.9 \pm 1.5) \times 10^{-5}$ s^{-1} ($R^2 = 0.993$ for 30 half-lives), yielding a k_{exch} of 9.81 ± 0.07 s^{-1}. The slight deviation from first-order fitting is due to the complexity of the integrated signal owing to several isotopomers such as CD_3, CD_2H, CDH_2, and CH_3. However, the intensity becomes zero as the exchange process

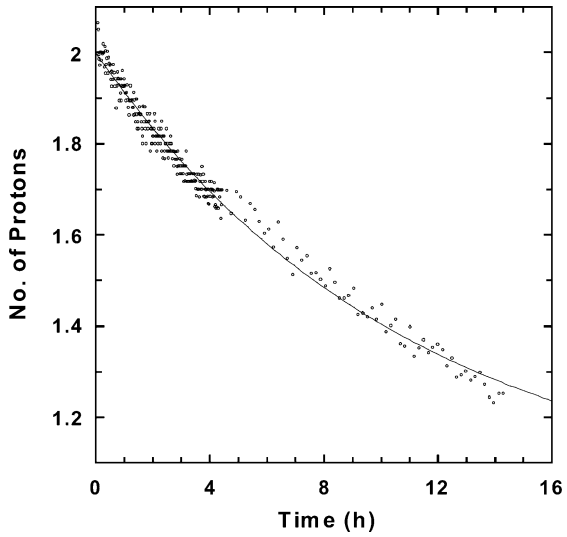


FIGURE 4: Kinetics of exchange of C-2 protons of *proteo*-glutaryl-CoA with wild-type GCD. The C-2 methylene hydrogens were monitored at 2.20 ppm in comparison to the pantetheine 6″-methylene resonance at 2.44 ppm as an internal standard. The integral of two for the zero time point is an extrapolated value. *proteo*-Glutaryl-CoA (0.84 mM) was incubated with 0.96 μM GCD in D_2O buffer at 25 °C (no turnover). The data were analyzed by the first-order equation.

goes to completion in several hours. It has been shown that the catalytic base, Glu367, of *Megasphaera elsdenii* short-chain acyl-CoA dehydrogenase catalyzes exchange of C-4 protons of crotonyl-CoA with solvent protons (*17*). Similarly, Glu370Gln GCD does not catalyze detectable exchange of 4^c methyl protons of crotonyl-CoA, indicating that the catalytic base mediates the exchange (Figure 3B). The exchange reaction supports our hypothesis that decarboxylation of glutaconyl-CoA yields a dienolate; the latter hypothesis had been proposed earlier based on the observation of a charge transfer species between a dienolate and oxidized flavin when glutaconyl-CoA was reacted with Glu370Gln GCD (*6*). This dienolate can be stabilized by the hydrogen bonding of the carbonyl oxygen of the acyl-CoA with the amide hydrogen of Glu370 and the 2′-hydroxyl of the FAD prosthetic group (*4*).

Hydrogen Exchange at C-2 and C-3 of Glutaryl-CoA with Solvent. The first chemical step in the catalytic turnover of GCD is the abstraction of a proton from the *pro-R* C-2 position of the substrate producing a carbanion that can be stabilized as an enolate (*18*). Since solvent has access to the catalytic site, it may be possible that the enzyme catalyzes the exchange of the C-2 proton of glutaryl-CoA even in the absence of turnover due to the formation of the C-2 anion or an enolate. Figure 4 shows the kinetics of exchange of the methylene hydrogens at C-2 (2.20 ppm) when 0.84 mM *proteo*-glutaryl-CoA was incubated with 0.96 μM GCD in D_2O buffer at 25 °C (no turnover). Exchange of the C-3 hydrogen was simultaneously monitored at 1.86 ppm (data not shown). The pseudo-first-order rate constant, k_{obs}, is (2.53 $\pm$ 0.11) $\times$ 10^{-5} s^{-1} (R^2 = 0.98 for 1.9 half-lives) for C-2 hydrogen exchange and (2.00 $\pm$ 0.07) $\times$ 10^{-5} s^{-1} (R^2 = 0.99 for 1.9 half-lives) for C-3 hydrogen exchange, yielding a k_{exch} = 0.022 $\pm$ 0.001 and 0.017 $\pm$ 0.001 s^{-1} at C-2 and C-3, respectively. Thus, the rate constants for hydrogen exchange at C-2 and C-3 are nearly identical. Only two hydrogens remain, one each at C-2 and C-3. This is consistent with the stereospecificity of dehydrogenation in the acyl-CoA dehydrogenase family: (i) *pro-R* C-2 hydrogen of the substrate abstracted as a proton by the catalytic base and (ii) *pro-R* C-3 hydrogen of the substrate transferred as a hydride to N(5) of the *re* face of FAD (*18*−*20*). These *pro-R* C-2 and C-3 hydrogens are labile and, therefore, exchangeable. The exchange reaction supports our hypothesis that deprotonation of 3-thiaglutaryl-CoA yields an enolate; the latter hypothesis had been proposed earlier based on the observation of a charge transfer species between an enolate and oxidized flavin when 3-thiaglutaryl-CoA was reacted with GCD (*2*). This enolate can be stabilized by the hydrogen bonding of the carbonyl oxygen of the acyl-CoA with the amide hydrogen of Glu370 and the 2′-hydroxyl of the FAD prosthetic group (*4*).

As with GCD, the short-chain acyl-CoA dehydrogenase from *M. elsdenii* exchanges one hydrogen each from the C-2 and C-3 positions of butyryl-CoA at equal rates (*7*, *21*). Medium-chain acyl-CoA dehydrogenase from a variety of mammalian sources catalyzes exchange at either C-2 only or C-2 and C-3; however, in the latter case, the rates are never equal (see references cited in ref *21*).

Protonation Following Decarboxylation under Single Turnover Conditions. The results shown in Figures 1 and 2 suggested the following possibilities: (A) the classically accepted intramolecular 1,3-prototropic shift proposed by Gomes and co-workers (*7*) might not be exclusive, or (B) rapid exchange of the protonated Glu370 [Glu370(H^+)] might occur so that Glu370 transfers a solvent-derived hydron rather than a substrate-derived hydron to crotonyl-CoA dienolate. Since incubation of crotonyl-CoA with enzyme leads to rapid exchange at the C-4 position, we performed a single turnover experiment by rapid-quench method. Single turnover of GCD with *2,2,4,4-d*$_4$-glutaryl-CoA, as substrate, and $FcPF_6$, as electron acceptor, monitored quantitatively by mass spectrometry, would provide direct evidence for assessing C-2 → C-4 proton transfer. In this experiment, the reaction between wild-type GCD (82 μM in syringe A) and *2,2,4,4-d*$_4$-glutaryl-CoA (0.2 mM in syringe B) in the presence of $FcPF_6$ (1.5 mM in syringe B) in 50 mM phosphate buffer, pH 7.60, at 25 °C was monitored between 40 and 260 ms, and the products were quantitatively analyzed by negative ion LC-ESI-MS. If the intramolecular 1,3-prototropic shift were operative, then incubation of GCD with *2,2,4,4-d*$_4$-glutaryl-CoA [HOOC−C(^{2}H)$_2$−CH$_2$−C(^{2}H)$_2$−C(=O)−SCoA; $C_{26}H_{38}(^2H)_4N_7O_{19}P_3S$; exact mass 885.2] and $FcPF_6$ would yield exclusively *2,4,4,4-d*$_4$-crotonyl-CoA [C(^{2}H)$_3$−CH=C(^{2}H)−C(=O)−SCoA; $C_{25}H_{36}(^2H)_4N_7O_{17}P_3S$; exact mass 839.2] in a single turnover. The mass spectral results are shown in Figure 5. Figure 5C shows the mass spectral profile of *proteo*-octanoyl-CoA used as an internal standard for quantitation of the substrate and product. The isotopic distribution of enzymatically generated crotonyl-CoA, at a reaction time of 260 ms, is shown in Figure 5A. The data indicate that the product is almost entirely d_3-crotonyl-CoA [*m*/*z* 837.2, [M − H^+]$^-$; $C_{25}H_{37}(^2H)_3N_7O_{17}P_3S$; exact mass 838.2]. The d_3-crotonyl-CoA species was by far most abundant (>97%) at all eight time points between 40 and 260 ms. *2,4,4,4-d*$_4$-Crotonyl-CoA was never observed in any significant amount (d_4-crotonyl-CoA < 3% of d_3-crotonyl-CoA; 3% is the limit of detection) at any time, indicating the involvement of solvent-derived protons in the

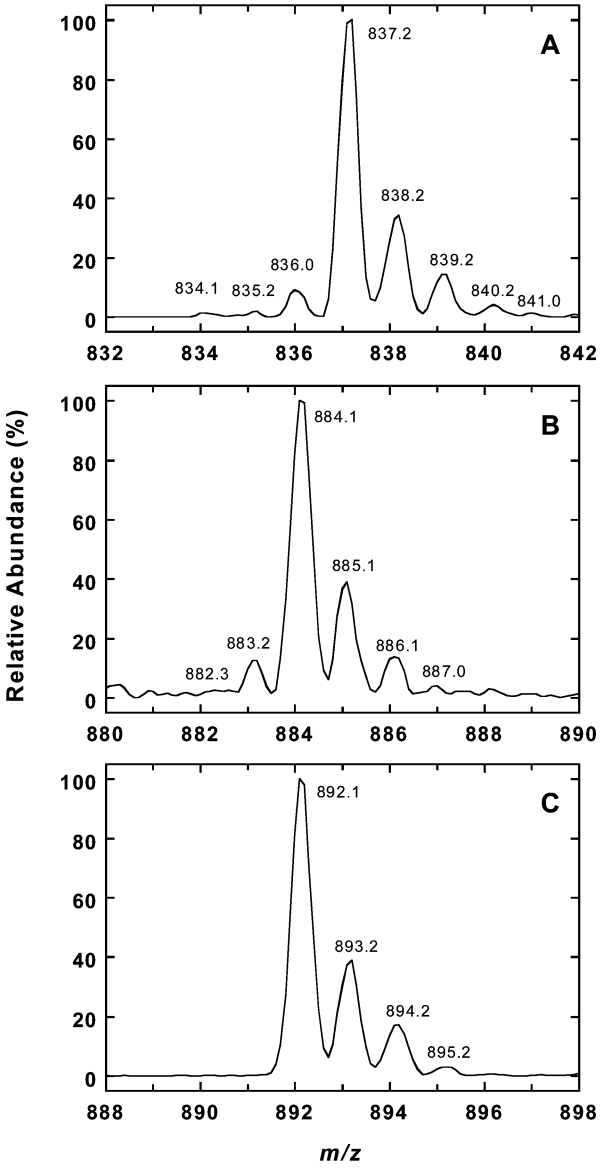


FIGURE 5: Negative ion ESI−mass spectrum of the 260 ms reaction between wild-type GCD and *2,2,4,4-d*$_4$-glutaryl-CoA with $FcPF_6$ in aqueous buffer as performed by the KinTek RQF-3 quench-flow apparatus. (A) Relative isotopic abundance, $[M - H^+]^-$, of the crotonyl-CoA product. From the observation of the major isotopic species of the crotonyl-CoA product (*m/z* 837.2, $[M - H^+]^-$) it is clear that the majority of the molecules carry three deuterons, i.e., d_3-crotonyl-CoA. Note the presence of d_2-crotonyl-CoA (*m/z* 836.0, $[M - H^+]^-$), which is consistent with the presence of d_3-glutaryl-CoA in the substrate (see below). (B) Relative isotopic abundance, $[M - H^+]^-$, of *2,2,4,4-d*$_4$-glutaryl-CoA. Notice the presence of a small amount of d_3-glutaryl-CoA (*m/z* 883.2, $[M - H^+]^-$) in the substrate. (C) Relative natural isotopic abundance, $[M - H^+]^-$, of *proteo*-octanoyl-CoA which was added to the stop-quench reaction as an internal standard for quantifying mass spectral data. In all of the samples the higher mass isotopic distributions (*m/z* > 837.2, d_3-crotonyl-CoA; *m/z* > 884.2, d_4-glutaryl-CoA; and *m/z* > 892.2, *proteo*-octanoyl-CoA) are representative of natural isotopic abundance of organic compounds containing C, H, N, O, P, and S.

oxidative decarboxylation of glutaryl-CoA by GCD. The *2,2,4,4-d*$_4$-glutaryl-CoA is >95% isotopically pure although a small percentage of d_3-glutaryl-CoA (*m/z* 883.2, $[M - H^+]^-$) is consistently seen (Figure 5B). Also, consistent with the presence of d_3-glutaryl-CoA in the starting material, we observe a comparable abundance of d_2-crotonyl-CoA (*m/z* 836.0, $[M - H^+]^-$) (Figure 5A).

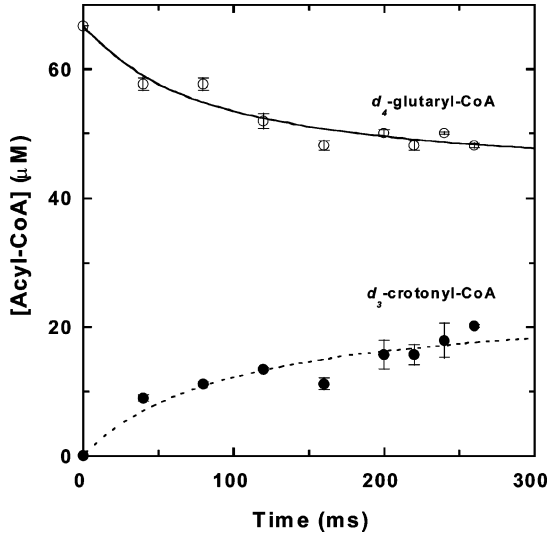


FIGURE 6: Progress of the oxidative decarboxylation of *2,2,4,4-d*$_4$-glutaryl-CoA by GCD in the presence of $FcPF_6$. *proteo*-Octanoyl-CoA was added after the reaction as an internal standard for quantitation of mass spectral data. The abundance of *2,2,4,4-d*$_4$-glutaryl-CoA, d_3-crotonyl-CoA, and *proteo*-octanoyl-CoA was measured by LC-ESI-MS SIM of specific, respective $[M - H^+]^-$ ions as described in Experimental Procedures. The data of consumption of *2,2,4,4-d*$_4$-glutaryl-CoA (○) and production of d_3-crotonyl-CoA (●) as a function of reaction time have been fitted to a first-order rate equation. The final concentrations of starting materials, GCD, *2,2,4,4-d*$_4$-glutaryl-CoA, and $FcPF_6$, are 27.3, 66.7, and 500 μM, respectively, at zero time.

It is now possible to quantify the reaction catalyzed by GCD by monitoring the progress of product formation (d_3-crotonyl-CoA) and substrate consumption (*2,2,4,4-d*$_4$-glutaryl-CoA) (Figure 6) because over the reaction time of 260 ms only d_3-crotonyl-CoA was observed. Even at the earliest time point, 40 ms, only d_3-crotonyl-CoA is observed with only a fraction of a turnover (~25%). At 260 ms, the amount of d_3-crotonyl-CoA observed remains lower than the concentration of enzyme sites, indicating that appropriate conditions were employed to monitor single turnover. The data may be analyzed as a first-order reaction with enzyme sites as the limiting species. The first-order rate constants, k_{obs}, for the depletion of substrate and formation of d_3-crotonyl-CoA product, are 11.6 ± 3.6 and 11.9 ± 2.9 s^{-1}, respectively (R^2 > 0.94 for both). The extent of the reaction is limited by (a) available enzyme sites occupied by substrate and (b) the reaction time. Based on enzyme sites, the maximum yield of crotonyl-CoA should be 23.2 μM resulting from (a) a 1:1 reaction between enzyme and substrate before being quenched, (b) the K_m of the substrate, as an approximation to the dissociation constant, to evaluate the occupancy of enzyme sites, and (c) the 3-fold dilution upon quenching. Experimentally, 19.1 ± 1.5 μM crotonyl-CoA formation is observed, i.e., 82% completion in 260 ms (Figure 6). The conditions used indicate that single turnover conditions are met satisfactorily. Therefore, the first-order rate constant, k_{obs}, probably reflects the slowest chemical step in the turnover of GCD among several chemical events that occur in the active site: (i) α-proton abstraction, (ii) β-hydride transfer, (iii) decarboxylation of glutaconyl-CoA, and (iv) protonation of crotonyl-CoA dienolate besides two $1e^-$ oxidations of reduced dehydrogenase FAD by $FcPF_6$. However, the single turnover condition precludes crotonyl-

CoA "product release" from the active site since the reaction is quenched before product is released. The identification of the rate-determining step in the overall turnover of GCD is currently under investigation. Under the above conditions, the steady-state parameters for GCD with *2,2,4,4-d*$_4$-glutaryl-CoA as substrate are $k_{cat} = 3.87 \pm 0.06$ s^{-1} (expressed as a 2e$^-$ oxidation) and $K_m = 3.4 \pm 0.2$ μM.[2]

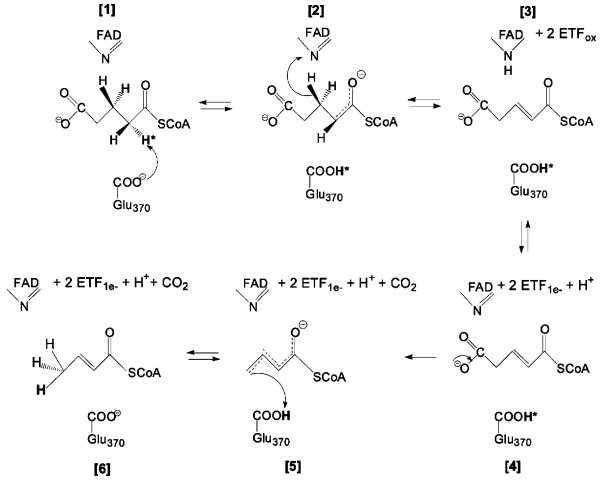
These data are consistent with a mechanism in which the dienolate is formed directly by decarboxylation. However, if the dienolate is not rapidly protonated at C-4 by Glu370(H$^+$) but instead is simply released upon quenching, a similar result could be obtained in the single turnover experiment. We have assumed that all of the chemical events are complete (except the physical event of product release) in the single turnover experiment. This appears to be the case based on two arguments. First, the rate constants of 11.6 or 11.9 s^{-1} imply that one complete set of chemical events must have occurred in roughly 80−90 ms. If there were a 1,3-prototropic shift occurring in this time scale (or up to 260 ms; see Figure 6), it would have been reflected in the mass spectral data. This was not the case; only *d*$_3$-crotonyl-CoA was observed in the single turnover experiment. Second, GCD with *proteo*-glutaryl-CoA as substrate shows a k_{cat} of about 5−6 s^{-1} (expressed as a 2e$^-$ oxidation). The pK_a of glutamic acid in the interior of a protein can lie between 4.5 and 8.0. Based on the pK_a, the maximal expected rate constant for the transfer of a proton from glutamic acid (carboxylic acid form) to water and from water to glutamate (carboxylate anionic form), k_{tr}, may be estimated to be $1-2 \times 10^3$ s^{-1} (*22*). The ratio of k_{cat}/k_{tr} ($5-6/1-2 \times 10^3$) suggests that one in every 167−400 turnovers would lead to a 1,3-prototropic shift without proton exchange with solvent. Gomes and co-workers, however, proposed exactly the opposite (*7*). In contrast, ketosteroid isomerase, known to be the most efficient enzyme, adopts a 1,3-prototropic shift because the proton exchange reaction with bulk solvent appears to be far slower than turnover (ref *23* and references cited therein). Thus, in case of GCD, the inclusion of the exchange process and the validity of the assumption that all of the chemical events are complete within the time scale of a single turnover seem justified.

Earlier we mentioned that *trans* C-2 and C-3 hydrogens of crotonyl-CoA do not exchange with solvent-derived hydrogens. The hydrogen exchange rate of C-2 and C-3 of substrate is only 0.2% that of C-4 of crotonyl-CoA. Thus, the contribution of hydrogen exchange at C-2 or C-3, in the single turnover experiment, is negligible. Therefore, the proton abstracted from the C-2 position of substrate by the catalytic base, Glu370, must exchange rapidly with solvent-derived protons before proton transfer to C-4 of the transient crotonyl-CoA dienolate. An unlikely alternative (based on k_{exch} of 9.8 s^{-1} of C-4 of crotonyl-CoA) is that the original α-proton is transferred to C-4, in a 1,3-prototropic shift, and then exchanges extremely rapidly with solvent protons.

DISCUSSION

Scheme 1 summarizes the results of the experiments presented in this paper. The single turnover experiment shows that the C-2 proton of substrate abstracted by Glu370 rapidly exchanges with solvent-derived protons and there is no detectable intramolecular transfer to C-4 following decarboxylation. Exchange must occur without dissociation of ligand from the active site since glutaconyl-CoA, the product of the reductive half-reaction, remains enzyme-bound (*6*). After decarboxylation of glutaconyl-CoA, the transient crotonyl-CoA dienolate forms a charge transfer complex ($\lambda_{max} \approx 725$ nm) with the oxidized isoalloxazine of FAD (*6*). Protonation of the dienolate by solvent-derived protons is observed as the decay of the charge transfer species (*6*). This previously unrecognized step in catalysis is the rapid exchange of the C-2 proton, which resides on the Glu370 carboxyl group, with solvent protons while the active site remains occupied with the glutaconyl-CoA reaction intermediate. Scheme 1 provides a rationale for the hydration of glutaconyl-CoA and crotonyl-CoA (*6*) and hydrolysis of the CoA thiolester from the covalent adduct following the covalent inactivation of GCD by 2-pentynoyl-CoA (*5*). Other members of the acyl-CoA dehydrogenase family also show low intrinsic hydratase activity (*24*, *25*) and are inactivated by 2-alkynoyl-CoAs, which involves cleavage of CoAS$^-$ from the covalent adduct (*26*); however, solvent is not envisaged to be involved in the catalytic pathways of these enzymes (*3*). In addition to the decarboxylation, the participation of solvent differentiates the mechanism of GCD from the other members of the acyl-CoA dehydrogenase family (*3*, *27*).

Scheme 1: Oxidative Decarboxylation of Glutaryl-CoA by Human GCD with Production of Crotonyl-CoA, H$^+$, and CO_2 as Products[a]



[a] Glu370 is the catalytic base in human GCD (*2*, *4*, *5*). Steps: [1] α-Proton abstraction by the catalytic base, Glu370, leads to an enolate (C-2 carbanion) with concomitant formation of the conjugate acid, Glu370(H$^+$). [2] Hydride transfer to the N(5) of FAD from the C-3 position leading to [3] 2e$^-$ reduced FAD with the enzyme bound, glutaconyl-CoA. [4] Reoxidation of 2e$^-$ reduced FAD by 2 equiv of ETF_{ox} in two steps leading to ETF_{1e^-} and initiation of the decarboxylation reaction upon partial reoxidation of FAD. [5] Formation of the dienolate (crotonyl-CoA anion) intermediate. [6] Protonation at the C-4 position by the conjugate acid, Glu370(H$^+$), by solvent-derived protons. Solvent-derived protons can exchange rapidly with the proton of the conjugate acid, Glu370(H$^+$), between steps [4], [5], and [6]. Exchange of γ-protons on crotonyl-CoA indicates that the step between [5] and [6] is reversible whereas breaking of the C4−C5 σ bond in the decarboxylation reaction (step between [4] and [5]) is irreversible. Decarboxylation of glutaconyl-CoA by the oxidized form of GCD is also depicted in steps [4], [5], and [6] (*6*). Gomes and co-workers had suggested a 1,3-prototropic shift in catalysis of GCD since it was held that the active site was shielded from bulk solvent (*7*).

[2] M. Albro, K. S. Rao, and F. E. Frerman, unpublished data.

The dienolate of crotonyl-CoA is most likely stabilized by hydrogen bonding of the thioester carbonyl oxygen with the backbone amide hydrogen of Glu370 and the 2′-hydroxyl of the ribityl side chain of FAD (*4*). Similar hydrogen bonding of the carbonyl oxygen of glutaconyl-CoA is observed in the crystal structure of *Acidaminococcus fermentans* glutaconyl-CoA decarboxylase (*28*). This decarboxylase is a subunit of a sodium ion pump that transduces the free energy of decarboxylation to generate a sodium ion gradient (*29*). The backbone amide hydrogens of Gly194 and Val151 function as hydrogen bond donors to the thioester carbonyl oxygen in the bacterial glutaconyl-CoA decarboxylase.

Dakoji and co-workers have shown that the reaction catalyzed by *M. elsdenii* short-chain acyl-CoA dehydrogenase involves a single base mechanism (*17*). Glu367 is the catalytic base in *M. elsdenii* short-chain acyl-CoA dehydrogenase and is required for proton exchange at the C-4 methyl of crotonyl-CoA (*17*). The similarity of active sites between GCD and short-chain acyl-CoA dehydrogenase (*4*, *27*) suggests that catalysis of C-4 proton exchange by GCD also involves a single base and that Glu370 initiates the proton abstraction at C-2 and transfers a proton to the C-4 position (*2*, *5*). We demonstrated the participation of Glu370 in proton transfer by the inability of the Glu370Gln GCD to catalyze exchange. The data presented here indicate that protonation of the transient crotonyl-CoA dienolate occurs exclusively by solvent-derived protons. Spectroscopic detection of the crotonyl-CoA dienolate is dependent on the mutation of Glu370 to Gln that significantly retards the decay of the spectral signal representing the dienolate (*6*). The catalytic base, Glu370, can apparently function as a conjugate acid [Glu370(H^+)] as does Glu164 in crotonase (*10*). In preliminary experiments, the charge transfer was not detected in rapid kinetic (stopped-flow spectrophotometry) experiments with the wild-type dehydrogenase; however, the charge transfer species can be detected with the Glu370Asp mutant of GCD.[2] Maximum absorbance of the charge transfer species with Glu370Asp GCD occurred at 4 s, which decayed rapidly. The maximum intensity of the charge transfer species reached only 28% of that observed with the Glu370Gln mutant before decay. Glutaconyl-CoA was not observed in our stop-quench experiments, suggesting that decarboxylation by wild-type GCD occurs very rapidly.

Gomes and co-workers (*7*) showed that the turnover of glutarylpantetheine in a ^3H_2O buffer by GCD from *Pseudomonas fluorescens* resulted in incorporation of less than 1 tritium from solvent per 200 turnovers. This result suggested that the active site is desolvated upon substrate binding and that the active site is shielded from water. Hence they proposed a 1 → 3 prototropic shift. In our experiments, extensive turnover of *2,2,4,4-d*$_4$-glutaryl-CoA by the human enzyme shows incorporation of solvent protons into crotonyl-CoA. This can be explained by the exchange of solvent protons on rebinding of the crotonyl-CoA product or exchange of the C-2 proton initially abstracted from the substrate. The enzyme catalyzes the exchange of C-4 protons at a rate of 9.8 s^{-1} at 25 °C, which is similar to the two-electron steady-state turnover at 25 °C, ≈5−6 s^{-1} (*30*). These data indicate that rebinding crotonyl-CoA can result in exchange of solvent protons at the C-4 methyl group and that crotonyl-CoA dissociation may be rate-determining in the steady state. Further, under single turnover conditions we demonstrated the involvement of water in the catalytic process. It is possible that the disagreement of our work with the work of Gomes and co-workers (*7*) may only be apparent because their investigation used pantetheine esters rather than CoA esters. Those alternate substrates may have a much shorter residence time in the active site to severely limit exchange (*31*, *32*).

There is no obvious pathway for water to enter or CO_2 to leave the active site of GCD. In the crystal structure of human GCD with 4-nitrobutyryl-CoA bound in the active site, there are no bound water molecules in the active site, and it is proposed that bound water molecules in the unliganded molecule are stripped when substrate is bound (*4*). Access of water is possible from the *si* face of FAD into the active site since hydrogen exchange occurs at C-4 of crotonyl-CoA and both C-2 and C-3 of substrate. Hydrogen exchange at C-3 must occur at the level of reduced FAD at the N(5)−H bond, which is very labile (*20*, *33*). Further, in medium-chain acyl-CoA dehydrogenase, N(5) of FAD forms a 2.9 Å hydrogen bond with Oγ−Thr168, which lies on the *si* face of FAD (*34*). Thr168 of medium-chain acyl-CoA dehydrogenase is a conserved residue among the acyl-CoA dehydrogenase family (*4*). A well-ordered and conserved water molecule in acyl-CoA dehydrogenases, W4, hydrogen bonds with the backbone carbonyl of Thr170 of human GCD (*4*). The backbone amide of Thr170 is partially exposed to bulk solvent (*4*). Conformational changes could occur upon ligand binding that may be responsible for the exchange processes. As noted by Taraphder and Hummer, an energy-minimized static structure may be inadequate to detect subtle proton transfer paths that depend on cooperative interactions of amino acid side chains and water molecules that connect the surface of the protein with a buried active site (*35*, *36*). Potential proton and water translocating pathways are typically truncated in crystal structures, suggesting dynamic pathways that result from fluctuations in the protein structure yield transient hydration of an otherwise hydrophobic cavity (*35*, *36*).

The other possibility is that the proton abstracted from either C-2 of substrate or C-4 of crotonyl-CoA equilibrates rapidly with a solvent-accessible pool that is not identifiable in the crystal structure. A pool of protons or water that can communicate between the catalytic base and external medium had been suggested in aconitase (*37*) while more recent structural studies implicate a hydrogen-bonding network to explain access of protons to the active site (*38*). Another example of the proposed pool of protons is in pyruvate kinase (*39*) whereas more recent structural studies suggest involvement of a water channel (*40*). In the case of GCD, neither a "water channel" nor a "pool of water/protons" is apparent in the crystal structure suggesting dynamic pathways as a possibility. A pathway responsible for the entry of water to the active site of GCD and its identification remain an open question.

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REFERENCES

1. Lenich, A. C., and Goodman, S. I. (1986) The purification and characterization of glutaryl-CoA dehydrogenase from porcine and human liver, *J. Biol. Chem. 261*, 4090−4096.
2. Dwyer, T. M., Rao, K. S., Goodman, S. I., and Frerman, F. E. (2000) Proton abstraction reaction, steady-state kinetics and oxidation−reduction potential of human glutaryl-CoA dehydrogenase, *Biochemistry 39*, 11488−11499.
3. Ghisla, S., and Thorpe, C. (2004) Acyl-CoA dehydrogenases: a mechanistic overview, *Eur. J. Biochem. 271*, 494−508.
4. Fu, Z., Wang, M., Paschke, R., Rao, K. S., Frerman, F. E., and Kim, J.-J. P. (2004) Crystal structures of human glutaryl-CoA dehydrogenase with and without an alternate substrate: structural bases of dehydrogenation and decarboxylation reactions, *Biochemistry 43*, 9674−9684.
5. Rao, K. S., Albro, M., Vockley, J., and Frerman, F. E. (2003) Mechanism-based inactivation of human glutaryl-CoA dehydrogenase by 2-pentynoyl-CoA: rationale for enhanced reactivity, *J. Biol. Chem. 278*, 26342−26350.
6. Westover, J. B., Goodman, S. I., and Frerman, F. E. (2001) Binding, hydration and decarboxylation of the reaction intermediate, glutaconyl-CoA by human glutaryl-CoA dehydrogenase, *Biochemistry 40*, 14106−14114.
7. Gomes, B., Fendrich, G., and Abeles, R. H. (1981) Mechanism of action of glutaryl-CoA and butyryl-CoA dehydrogenases. Purification of glutaryl-CoA dehydrogenase, *Biochemistry 20*, 1481−1490.
8. Simon, E. J., and Shemin, D. (1953) The preparation of S-succinyl-CoA, *J. Am. Chem. Soc. 75*, 2520.
9. Corkey, B. E. (1988) Analysis of acyl-coenzyme A esters in biological samples, *Methods Enzymol. 166*, 55−70.
10. Hofstein, H. A., Feng, Y., Anderson, V. E., and Tonge, P. J. (1999) Role of glutamate 144 and glutamate 164 in the catalytic mechanism of enoyl-CoA hydratase, *Biochemistry 38*, 9508−9516.
11. Wlassics, I. D., and Anderson, V. E. (1989) Citrate synthase stabilizes the enethiolate of acetyldithio coenzyme A, *Biochemistry 28*, 1627−1633.
12. Johnson, K. A. (1995) Rapid quench kinetic analysis of polymerases, adenosinetriphosphatases, and enzyme intermediates, *Methods Enzymol. 249*, 38−61.
13. Dunaway-Mariano, D. (1999) Intermediate and energetics in pyruvate phosphate dikinase, *Methods Enzymol. 308*, 149−176.
14. Bökenkamp, D., Desai, A., Yang, X., Tai, Y.-C., Marzluff, E. M., and Mayo, S. L. (1998) Microfabricated silicon mixers for submillisecond quench-flow analysis, *Anal. Chem. 70*, 232−236.
15. Barman, T. E., and Gutfreund, H. (1964) A comparison of the resolution of chemical and optical sampling, in *Rapid Mixing and Sampling Techniques in Biochemistry* (Chance, B., Gibson, Q. H., Eisenhardt, R. H., and Lonberg-Holm, K. K., Eds.) pp 339−343, Academic Press, New York.
16. Wu, W.-J., Tonge, P. J., and Raleigh, D. P. (1998) Stereospecific ^{1}H and ^{13}C NMR assignments of crotonyl-CoA and hexadienoyl-CoA: conformational analysis and comparison with protein-CoA complexes, *J. Am. Chem. Soc. 120*, 9988−9994.
17. Dakoji, S., Shin, I., Becker, D. F., Stankovich, M. T., and Liu, H.-w. (1996) Studies of acyl-CoA dehydrogenase catalyzed allylic isomerization: a one-base or two-base mechanism, *J. Am. Chem. Soc. 118*, 10971−10979.
18. Ghisla, S., and Thorpe, C. (2004) Acyl-CoA dehydrogenases. A mechanistic review, *Eur. J. Biochem. 271*, 494−508.
19. Kim, J.-J. P., and Wu, J. (1992) Three-dimensional structure of medium-chain acyl-CoA dehydrogenase, in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. 3, pp 299−307, CRC Press, Boca Raton, FL.
20. Pai, E. F. (1992) The stereochemistry of the prosthetic groups of flavoproteins, in *Chemistry and Biochemistry of Flavoenzymes* (Müller, F., Ed.) Vol. 3, pp 357−366, CRC Press, Boca Raton, FL.
21. Ghisla, S., Thorpe, C., and Massey, V. (1984) Mechanistic studies with general acyl-CoA dehydrogenase and butyryl-CoA dehydrogenase: Evidence for the transfer of the β-hydrogen to the flavin N(5)-position as a hydride, *Biochemistry 23*, 3154−3161.
22. Fersht, A. R. (1999) *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*, pp 132−190, W. H. Freeman, New York.
23. Pollack, R. M. (2004) Enzymatic mechanisms for catalysis of enolization: ketosteroid isomerase, *Bioorg. Chem. 32*, 341−353.
24. Lau, S.-M., Powell, P., Buettner, H., Ghisla, S., and Thorpe, C. (1986) Medium chain acyl-CoA dehydrogenase from pig kidney has intrinsic enoyl-CoA hydratase activity, *Biochemistry 25*, 4184−4189.
25. Ellison, P. A., and Engel, P. C. (1993) Intrinsic crotonase activity in bacterial butyryl-CoA dehydrogenase, *Biochem. Mol. Biol. Int. 29*, 605−612.
26. Freund, K., Mizzer, J., Dick, W., and Thorpe, C. (1985) Inactivation of general acyl-CoA dehydrogenase from pig kidney by 2-alkynoyl-coenzymeA derivatives: initial aspects, *Biochemistry 24*, 5996−6002.
27. Kim, J.-J. P., Wang, M., Paschke, R., and Roberts, D. (1999) Evolution of an active site: The three-dimensional structures of acyl-CoA dehydrogenases, in *Flavins and Flavoproteins 1999* (Ghisla, S., Kroneck, P., Macheroux, P., and Sund, H., Eds.) pp 491−498, Agency for Scientific Publications, Berlin.
28. Wendt, K. S., Schall, I., Huber, R., Buckel, W., and Jacob, U. (2003) Crystal structure of the carboxyltransferase subunit of the bacterial sodium ion pump glutaconyl-CoA decarboxylase, *EMBO J. 22*, 3493−3502.
29. Buckel, W. (2001) Sodium translocating decarboxylases, *Biochim. Biophys. Acta 1505*, 15−27.
30. Rao, K. S., Vander Velde, D., Dwyer, T. M., Goodman, S. I., and Frerman, F. E. (2002) Alternate substrates of human glutaryl-CoA dehydrogenase: structure and reactivity of substrates and identification of a novel 2-enoyl-CoA product, *Biochemistry 41*, 1274−1284.
31. Bahnson, B. J., and Anderson, V. E. (1989) Isotope effects on the crotonase reaction, *Biochemistry 28*, 4173−4181.
32. Bahnson, B. J., and Anderson, V. E. (1991) Crotonase-catalyzed β-elimination is concerted: A double isotope effect study, *Biochemistry 30*, 5894−5906.
33. Manstein, D. J., Pai, E. F., Schopfer, L. M., and Massey, V. (1986) Absolute stereochemistry of flavins in enzyme-catalyzed reactions, *Biochemistry 25*, 6807−6816.
34. Fraaije, M. W., and Mattevi, A. (2000) Flavoenzymes: diverse catalysts with recurrent features, *Trends Biochem. Sci. 25*, 126−132.
35. Taraphder, S., and Hummer, G. (2003) Dynamic proton transfer pathways in proteins: role of side chain conformational fluctuations, *Physics A 318*, 293−301.
36. Taraphder, S., and Hummer, G. (2003) Protein side-chain motion and hydration in proton transfer pathways. Results for cytochrome P450cam, *J. Am. Chem. Soc. 125*, 3931−3940.
37. Kuo, D. J., and Rose, I. A. (1987) Aconitase: its source of catalytic protons, *Biochemistry 26*, 7589−7596.
38. Lauble, H., Kennedy, M. C., Beinert, H., and Stout, C. D. (1992) Crystal structure of aconitase with isocitrate and nitroisocitrate bound, *Biochemistry 31*, 2735−3748.
39. Rose, I. A., Kuo, D. J., and Warms, J. V. B. (1991) A rate-determining proton relay in the pyruvate kinase reaction, *Biochemistry 30*, 722−726.
40. Susan-Resiga, D., and Nowak, T. (2003) The proton transfer step catalyzed by yeast pyruvate kinase, *J. Biol. Chem. 278*, 12660−12671.

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