Alternate Substrates of Human Glutaryl-CoA Dehydrogenase: Structure and Reactivity of Substrates, and Identification of a Novel 2-Enoyl-CoA Product†

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ABSTRACT: The dehydrogenation reaction catalyzed by human glutaryl-CoA dehydrogenase was investigated using a series of alternate substrates. These substrates have various substituents at the γ position in place of the carboxylate of the physiological substrate, glutaryl-CoA. The steady-state kinetic constants of the six alternate substrates and the extent of flavin reduction in the anaerobic half-reaction were determined. One of these substrates, 4-nitrobutyryl-CoA, was previously thought not to be a substrate of the dehydrogenase; however, the enzyme does oxidize this substrate analogue with a k_{cat} that is less than 2% of that with glutaryl-CoA when ferrocenium hexafluorophosphate ($FcPF_6$) is the electron acceptor. Anaerobic titration of the dehydrogenase with 4-nitrobutyryl-CoA showed no reduction of the flavin; but instead showed an increased absorbance in the 460 nm region suggesting deprotonation of the analogue to form the α-carbanion. Analysis of these data indicated a binding stoichiometry of about 1.0. Under aerobic conditions, a second absorption maximum is observed with $\lambda_{max} = 366$ nm. The generation of the latter chromophore is dependent on an electron acceptor, either O_2 or $FcPF_6$, and is greatly facilitated by the catalytic base Glu370. The 466 nm absorbing species remains enzyme-bound while the 366 nm absorbing species is present only in solution. The latter compound was identified as 4-nitronate-but-2-enoyl-CoA by mass spectrometry, ^{1}H NMR, and chemical analyses. Ionization of the enzymatic product, 4-nitro-but-2-enoyl-CoA, that yields the nitronate occurs in solution and not on the enzyme. The variation of k_{cat} with the nature of the substituent suggests that the various substituents affect the free energy of activation, $\Delta G^{\ddagger}$, for dehydrogenation. There is a good correlation between log(k_{cat}) and F, the field effect parameter, of the γ-substituent. No correlation was found between any other kinetic or equilibrium constants and the substituent parameters using quantitative structure−activity relationships (QSAR). 4-Nitrobutyryl-CoA is the extreme example with the strongly electron-withdrawing nitro group in the γ position.

Glutaryl-CoA dehydrogenase (GCD)[1] catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA and CO_2 (*1*). Substrate oxidation and decarboxylation are not obligatorily coupled, and some acyl-CoA thioesters, e.g., pentanoyl-, hexanoyl-, and glutaramyl-CoA, that cannot be decarboxylated, are good alternate substrates of the human enzyme (*2*, *3*). Also, Gomes et al. showed that the γ-methyl ester of glutaryl-CoA is a substrate of *Pseudomonas fluorescens* GCD (*4*). Alternate substrates and substrate analogues are often useful reagents for enzyme mechanistic studies. Byron and colleagues recently reported the synthesis of 4-nitrobutyryl-CoA as a potential substrate analogue for GCD (*5*). Nitro compounds are excellent analogues for the related carboxylic acids. The nitro group is planar like the carboxylate group and has an extremely high dipole moment, essentially identical to those of carboxylic acids and amides, and about 2-fold greater than the corresponding esters. The strong inductive effect of the nitro group decreases the pK_a of adjacent methylene protons, and inductive effects could make hydride transfer from the β-carbon of glutaryl-CoA less favorable. These properties suggested that 4-nitrobutyryl-CoA could be a potentially useful dead-end inhibitor since it was previously reported that 4-nitrobutyryl-CoA was not a substrate of human GCD (*5*). Further, some nitro substrate analogues function as active site directed inhibitors that bind covalently and irreversibly at the active sites (*6*). Nitro analogues, as nitronate anions, may also serve as transition-state analogues of carbanionic intermediates (*7*).

Medium-chain acyl-CoA dehydrogenase is the archetype of the acyl-CoA dehydrogenases (*8*). The design of alternate substrates, substrate analogues, and active site directed

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[1] Abbreviations: GCD, glutaryl-CoA dehydrogenase; $FcPF_6$, ferrocenium hexafluorophosphate; QSAR, quantitative structure−activity relationships.

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inhibitors for this dehydrogenase has focused on the properties of analogues that demonstrate the acidification of α-protons, the redistribution of charge during catalysis, and, more recently, charge properties of the β-carbon that are related to modulation of flavin redox potential and development of the transition state for the dehydrogenase (*9*−*14*). In the experiments described in this work, several alternate substrates of glutaryl-CoA dehydrogenase have been examined that focus on substituents at the γ-carbon that are substituted for a carboxylate in the physiological substrate, glutaryl-CoA. The experiments consider the effects of the interaction of the dehydrogenase with the γ-substituent, and field-inductive effects of the substituents. Previous experiments show that electrostatic catalysis is an important element in the catalytic pathway of glutaryl-CoA dehydrogenase and that Arg94 plays a critical role in this regard in the low dielectric constant of the active site (*3*). Unlike a previous study (*5*), we show that 4-nitrobutyryl-CoA is a substrate of the dehydrogenase, albeit a very poor one. 4-Nitrobutyryl-CoA can also be oxidized by an oxidase activity of the enzyme. The oxidase/dehydrogenase ratio of human GCD is about 75-fold greater with 4-nitrobutyryl-CoA than with glutaryl-CoA as substrate. The product of enzymatic oxidation of 4-nitrobutyryl-CoA is a novel acyl-CoA that exhibits an additional absorption maximum at 366 nm besides the usual 260 nm peak. This product was identified as 4-nitronate-but-2-enoyl-CoA by chemical and spectral methods. The low activity of GCD with 4-nitrobutyryl-CoA prompted examination of the effect of other substrates with γ-substituents on the kinetic properties of the enzyme. Substituents were chosen to address structure/activity relationships that could be mediated through the acyl chain and how each substituent affected substrate binding and dehydrogenation.

EXPERIMENTAL PROCEDURES

Materials. Ferrocenium hexafluorophosphate ($FcPF_6$), monomethyl-glutarate, and 4-nitrobutyric acid methyl ester were obtained from Aldrich. 5-Hexenoic acid was obtained from Lancaster. Deuterium oxide, CoASH, hexanoyl-CoA, glutaryl-CoA, protocatechuic acid, protocatechuate-3,4-dioxygenase, and horseradish peroxidase were purchased from Sigma. Protocatechuate-3,4-dioxygenase from *B. cepacia* DB01 was a generous gift of Professor David Ballou, University of Michigan, Ann Arbor. Amplex red reagent was obtained from Molecular Probes. Pentanoyl-CoA and glutaramyl-CoA were synthesized as previously described (*2*, *3*).

Enzymes. Wild-type GCD ($\epsilon_{447nm} = 14.5$ mM^{-1} cm^{-1}) and Glu370Asp ($\epsilon_{446nm} = 14.3$ mM^{-1} cm^{-1}), Glu370Gln ($\epsilon_{447nm} = 13.2$ mM^{-1} cm^{-1}), and Arg94Gly ($\epsilon_{447nm} = 14.2$ mM^{-1} cm^{-1}) GCD mutants were expressed in *Escherichia coli* and purified as previously described (*2*, *3*).

Enzyme Assays. Glutaryl-CoA dehydrogenase activity was routinely assayed at 25 °C in 10 mM Tris-HCl, pH 8.0, 30 μM glutaryl-CoA, and 200 μM $FcPF_6$ as the electron acceptor, using $\epsilon_{300nm} = 4.3$ mM^{-1} cm^{-1} (*2*). In steady-state kinetic experiments, the acyl-CoA was the varied substrate, and the steady-state constants were determined by nonlinear least-squares fit to the Michaelis−Menten equation.

Oxidase activity of GCD was measured by monitoring the increase in absorbance at 570 nm ($\epsilon_{570nm} = 54$ mM^{-1} cm^{-1}) with 22 μM Amplex red reagent and 6 units of horseradish peroxidase and glutaryl-CoA or 4-nitrobutyryl-CoA as substrate (*15*). This assay requires continuous stirring of the reaction mixture to obtain reproducible results. Amplex red reagent has been used for fluorometric determination of oxidases because of its high sensitivity. However, the absorbance method used here is also extremely sensitive due to the high molar absorptivity of the dye product, resorufin. There is no interference from components such as phenol in other oxidase assays (*16*, *17*). Oxidase activity was also assayed by measuring the increase in absorbance at 366 nm due to formation of the 4-nitronate-but-2-enoyl-CoA from 4-nitrobutyryl-CoA. The molar extinction coefficient, $\epsilon_{366nm} = 14.5$ mM^{-1} cm^{-1}, was determined by titrating $FcPF_6$ with known concentrations of 4-nitrobutyryl-CoA in the presence of 100 nM GCD, and monitoring the increase in absorbance at 366 nm. The change in absorbance at 366 nm was a linear function of the concentration of 4-nitrobutyryl-CoA added until the dye was completely reduced.

Synthesis of CoA Thioesters. 4-Nitrobutyric acid was prepared by alkaline hydrolysis of the methyl ester in water at 50−60 °C for 15 min with 1 equiv of NaOH. All acyl-CoA thioesters were synthesized by the mixed anhydride method (*18*). Acyl-CoA thioesters were purified by anion exchange chromatography on a column (25 × 180 mm) of DEAE-cellulose eluted with a linear gradient (1200 mL) of 0−250 mM LiCl in 1 mM HCl. Alternatively, the compounds were purified by high-performance liquid chromatography on a semipreparative, reversed-phase C-18 column (10 × 250 mm) which was eluted isocratically with 50 mM potassium phosphate buffer, pH 5.3, and methanol (95:5, v/v). The purified acyl-CoA thioesters were desalted on a column (10 × 900 mm) of Sephadex G-10 eluted with water. The CoA esters were stored lyophilized or frozen at −20 °C and were quantitated using $\epsilon_{260nm} = 16.4$ mM^{-1} cm^{-1} (*19*). The purity of the synthetic compounds was ≥98% as demonstrated by analytical high-performance liquid chromatography (*20*). Mass spectrometry of synthetic 4-nitrobutyryl-CoA (acid form, nonisotopic, $C_{25}H_{41}N_8O_{19}P_3S$, MW = 882.2) showed a single compound with an ion at *m*/*z* 883.3 ($[M+H]^+$) as expected. One other species ($[M+H]^+$, *m*/*z* 840.4) was also observed (<2%). This compound is tentatively identified as 3-hydroxy-propanoyl-CoA. The acyl group is a side product generated during alkaline hydrolysis of 4-nitrobutyric acid methyl ester. Further, the identity of 4-nitrobutyryl-CoA was confirmed by ^1H NMR (500 MHz, D_2O) and COSY. The typical chemical shifts were: δ 8.46 (s, 1H, H8), 8.18 (s, 1H, H2), 6.06 (d, $J = 6.6$ Hz, 1H, H1′), 4.77 (partly obscured by HDO, 1H, H2′), 4.70 (obscured by HDO, 1H, H3′), 4.50 (br, 1H, H4′), 4.46 (t, $J = 6.7$ Hz, 2H, H4‴), 4.15 (br, 2H, H5′), 3.93 (s, 1H, H3″), 3.74 and 3.47 [dd, $J = 4.9$, 9.9 Hz (coupling to ^{31}P), 1H each, H1″], 3.36 (t, $J = 6.0$ Hz, 2H, H5″), 3.24 (t, $J = 5.5$ Hz, 2H, H8″), 2.90 (t, $J = 5.5$ Hz, 2H, H9″), 2.66 (t, $J = 7.2$ Hz, 2H, H2‴), 2.34 (t, $J = 6.0$ Hz, 2H, H6″), 2.19 (pentet, $J = 7.0$ Hz, 2H, H3‴), 0.80 (s, 3H, H10″), and 0.67 (s, 3H, H11″). The phosphates, hydroxyls, amine, and amides are not observed since they are exchangeable.

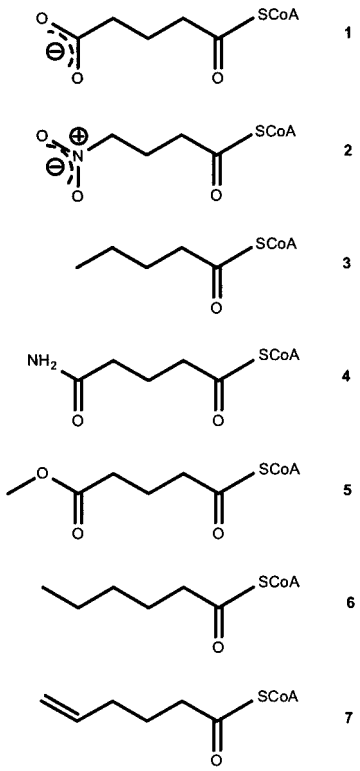
NMR and Mass Spectrometry. Enzymatic oxidation of 4-nitrobutyryl-CoA generated an unusual acyl-CoA product with an absorption maximum at 366 nm. This product was identified as follows: GCD was concentrated and the buffer

changed by chromatography on a column (2 mL) of DEAE-Sepharose (Fast Flow). GCD was adsorbed to the column and washed with 20 volumes of phosphate buffer, 20 mM, pH 7.8, followed by a wash with 5 volumes of the same buffer in D_2O. This step removed the glycerol in which the enzyme was stored and introduced the D_2O-containing buffer. The dehydrogenase was then eluted with potassium phosphate, 250 mM, pH 7.8 (pD 8.2), containing 500 mM KCl in D_2O. One milliliter of 100 μM enzyme was added to a known quantity of lyophilized 4-nitrobutyryl-CoA to bring the concentration of the acyl-CoA to 1 mM. The generation of the 366 nm absorbing chromophore was monitored spectrophotometrically using a 1 mm path length cuvette. When the absorbance at 366 nm reached a maximum, the enzyme was removed using a Centricon centrifugal membrane filter (YM-30) that had been prewashed with deuterated buffer. The filtrate was then analyzed by NMR spectroscopy. NMR spectra were taken on a Bruker Avance DRX 500 spectrometer operating at 500.13 MHz for ^{1}H. One- and two-dimensional spectra were recorded with standard Bruker pulse programs.

Samples for analysis by mass spectrometry were generated aerobically by incubation of 50 μM 4-nitrobutyryl-CoA with 5 μM glutaryl-CoA dehydrogenase in 10 mM ammonium formate, pH 8.1, in the presence and absence of 200 μM $FcPF_6$. When product formation reached a maximum, as judged by the absorbance at 366 nm, the enzyme was removed as above and the sample analyzed by mass spectrometry. Electrospray ionization mass spectra were obtained with a Perkin-Elmer Sciex API-3000 triple quadrupole mass spectrometer with a turbo ionspray source, interfaced with Perkin-Elmer HPLC series 200 system. Samples were analyzed in the flow injection mode with a Phenomenex C-18 guard column in isocratic 75% methanol in water containing 10 mM ammonium acetate at a flow rate of 200 μL/min and sample injection volume of 20 μL. The mass spectrometer settings were as follows: turbo ion spray temperature, 300 °C; needle spray, 4500 V; declustering potential, 30 V; focus plate, 175 V. Mass spectra were acquired from Q1 in either the positive or the negative ion mode, scanning the mass range from 200 to 1200 amu every 3 s.

Other Analytical Methods. Spectrophotometric titrations and determination of the kinetics of formation of the 466 and 366 nm absorbing species derived from 4-nitrobutyryl-CoA were performed on Hewlett-Packard diode array spectrophotometer, model 8452A or 8453. Steady-state kinetic assays were performed on a Shimadzu UV-2401 PC dual-beam spectrophotometer. Spectrophotometric titration data were analyzed using KaleidaGraph 3.5 software as described earlier (*2*, *3*). Anaerobic conditions were established as follows: One milliliter cuvettes were sealed with rubber septa after having been filled with a buffer that had been bubbled with argon for at least 20 min. Enzyme and 4 mM protocatechuate were added, and the cuvette was evacuated and purged with argon for 15 cycles. Protocatechuate dioxygenase (0.4 unit) was added with a gastight syringe and incubated for 15 min at 22 °C (*21*). The substrate was made anaerobic by evacuation and purging with argon for 15 cycles, and the enzyme was then titrated using a gastight syringe that was left inserted throughout the titration just at the surface of the liquid.

Chart 1: Acyl-CoAs Used in This Work[a]



[a] The compounds are as follows: **1**, glutaryl-CoA; **2**, 4-nitrobutyryl-CoA; **3**, pentanoyl-CoA; **4**, glutaramyl-CoA; **5**, monomethyl glutaryl-CoA; **6**, hexanoyl-CoA; **7**, 5-hexenoyl-CoA.

Structure−Activity Relationships. Quantitative structure−activity relationships (QSAR) were evaluated using the following equation:

$$\log(k_{cat}) = \rho F + c$$

where F is the field-effect parameter, ρ is the coefficient of F (also referred to as the reaction constant), and c is a constant. The experimental data are plotted against F values for each substituent obtained from Tables 13-10 in Hansch and Leo (*22*). We also analyzed the data by multivariate analyses; however, no better correlation of the data was obtained than the equation above. The substituents were chosen to include a wide range of F values yet be compatible with the restraints of the active site. The structures of these compounds are given in Chart 1.

RESULTS AND DISCUSSION

Steady-State Turnover of Glutaryl-CoA Analogues. The data in Table 1 show the steady-state kinetic constants of human GCD with glutaryl-CoA and six alternate substrates substituted at the γ-carbon (the structures are given in Chart 1). The turnover of the dehydrogenase with these alternate substrates falls into several groups. Hexanoyl-CoA and 5-hexenoyl-CoA are oxidized at rates similar to glutaryl-CoA. Pentanoyl-CoA and the 5-methyl ester of glutaryl-CoA are oxidized at about half the rate of glutaryl-CoA, and glutaramyl-CoA at about 20% the rate of glutaryl-CoA. In contrast, the k_{cat} with 4-nitrobutyryl-CoA as substrate is less than 2% that of glutaryl-CoA. Similar categories are evident when the substrates are grouped based on k_{cat}/K_m; the value of this apparent second-order rate constant with 4-nitrobu-

Table 1: Steady-State Kinetic Constants of Human Glutaryl-CoA Dehydrogenase with Glutaryl-CoA and Alternate Substrates[a]

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (μM^{-1} s^{-1})
glutaryl-CoA	12.8 ± 0.3	3.7 ± 0.3	3.50
glutaramyl-CoA	2.7 ± 0.2	6.6 ± 2.1	0.41
methyl-glutaryl-CoA	8.0 ± 0.6	20.1 ± 5.1	0.40
pentanoyl-CoA	6.3 ± 0.6	23.5 ± 7.5	0.27
hexanoyl-CoA	10.0 ± 0.7	85.1 ± 14.6	0.12
5-hexenoyl-CoA	13.1 ± 0.7	105 ± 13	0.12
4-nitrobutyryl-CoA	0.24 ± 0.01	14.1 ± 1.9	0.02

[a] Assayed spectrophotometrically in 10 mM Tris-HCl, pH 8.0 at 25 °C, with 200 μM $FcPF_6$ as the electron acceptor.

tyryl-CoA is less than 1% that with glutaryl-CoA. Using medium-chain acyl-CoA dehydrogenase with bound substrate as a model, glutaryl-CoA has been modeled into the active site of human GCD so that the thioester carbonyl oxygen of glutaryl-CoA hydrogen-bonds with the 2′-hydroxyl of FAD and the amide HN of Glu370. Under these conditions, the carboxylate of glutaryl-CoA is within hydrogen-bonding distance of Arg94 at the base of the active site (*23*). The interaction of Arg94 with glutaryl-CoA or any other charged substrate would represent an ionized hydrogen bond or salt-bridge. Our previous work also showed that Arg94 is important in the oxidation of glutaryl-CoA, but not alternate substrates with ethyl and amide γ-substituents (*3*). There seems to be no reason that the thioester oxygen of 4-nitrobutyryl-CoA could not hydrogen-bond with the peptidic amide hydrogen of Glu370 and the 2′-hydroxyl of FAD as is the case in other acyl-CoA dehydrogenases (*23*−*25*). The close steric and electronic relationship between the nitro analogue and glutaryl-CoA would also suggest that the nitro analogue has the capacity to hydrogen-bond with Arg94. However, 4-nitrobutyryl-CoA is a very poor substrate. In fact, alternate substrates without a polar γ-substituent are significantly better substrates whether judged by the value of k_{cat} or k_{cat}/K_m.

Reduction of Glutaryl-CoA Dehydrogenase with Glutaryl-CoA Analogues. Despite the close structural and electronic similarity of 4-nitrobutyryl-CoA and glutaryl-CoA, the analogue is a very poor substrate. The extent of flavin reduction under anaerobic conditions was determined by spectrophotometric titration. Anaerobic titration of glutaryl-CoA dehydrogenase with 4-nitrobutyryl-CoA shows no bleaching of the flavin chromophore (Figure 1); however, a low-level reduction of the flavin may be masked by almost a 2-fold increase in the absorbance at 447 nm. The absorption maximum in this region is also red-shifted to 466 nm. There is a small increase in absorbance in the region of the near-UV transition at 370 nm. However, some of this latter increase may be due to end absorbance of the acyl-CoA ligand in the single-beam instrument. When the mutant GCD, Glu370Gln, which lacks the catalytic base, was titrated with the nitro analogue under anaerobic conditions, there was no increase in absorbance at 466 nm (data not shown, but see Figure 2). The change in absorbance in the 466 nm region is similar to that observed by Vock et al. when the chromogenic transition-state analogue *p*-nitrophenylacetyl-CoA is bound by medium-chain acyl-CoA dehydrogenase (*26*). In that case, the increase in absorbance in the 460 nm region of the flavin was attributed to the anionic form of the ligand after deprotonation at the α-carbon, and perturbation of the flavin chromophore by that anion. Long-

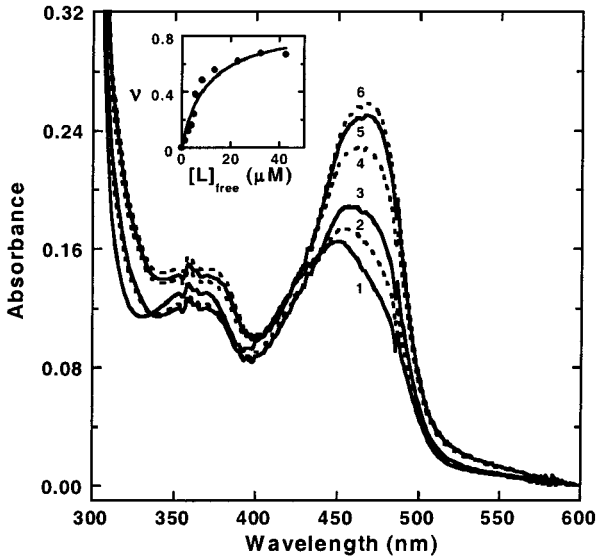


FIGURE 1: Spectrophotometric titration of wild-type glutaryl-CoA dehydrogenase with 4-nitrobutyryl-CoA under anaerobic conditions. The dehydrogenase, 11.4 μM (curve 1), in 50 mM potassium phosphate, pH 7.6, 10 °C, was titrated with 4-nitrobutyryl-CoA at the following concentrations (μM): (2) 4; (3) 8; (4) 14; (5) 30; and (6) 50; some spectra are omitted for clarity. The inset shows the binding isotherm from the titration. Data are analyzed at 474 nm using $\Delta\epsilon_{474nm} = 16.1$ mM^{-1} cm^{-1} as described by Dwyer et al. (*2*). The curve is fit to the data to yield a binding stoichiometry $n = 0.89 \pm 0.09$ and a dissociation constant $K_d = 10.5 \pm 2.6$ μM.

wavelength absorption (~750 nm) of the binary complex of medium-chain acyl-CoA dehydrogenase with *p*-nitrophenylacetyl-CoA was attributed to a charge-transfer species between the oxidized flavin and an anion that could be delocalized over the aromatic ring. No absorption at longer wavelengths, up to 900 nm, is observed when 4-nitrobutyryl-CoA is mixed with GCD. Taken together, the data suggest that the 466 nm absorbing species is generated by deprotonation at the α-carbon by the catalytic base, Glu370.

Under anaerobic conditions and without an electron acceptor such as electron-transfer flavoprotein or $FcPF_6$, there is no detectable reduction of the flavin, and no significant turnover (see below). Hence, from an anaerobic titration of GCD with 4-nitrobutyryl-CoA (Figure 1 and inset), we calculated a dissociation constant and binding stoichiometry using the absorbance changes at 474 nm wherein maximum changes were seen. The K_d, 10.5 ± 2.6 μM, at pH 7.6, is similar to the kinetic K_m at pH 8 which is 14.1 ± 1.9 μM (Table 1). The binding stoichiometry is 0.9 ± 0.1 per flavin.

Addition of a 10-fold molar excess of 4-nitrobutyryl-CoA to wild-type GCD under aerobic conditions at 5 °C resulted in a rapid increase in absorbance at 466 nm (Figure 2A). There was a slower time-dependent increase in absorbance at 366 nm. Identical experiments were carried out at 5 °C with the wild-type dehydrogenase under anaerobic conditions (Figure 2B), and several mutant dehydrogenases (Glu370Gln, Glu370Asp, and Arg94Gly, Figure 2C−E). The increased absorbances at 366 and 466 nm are very small, and the rates are considerably decreased under anaerobic conditions with the wild-type dehydrogenase, and under aerobic conditions with the Glu370Gln mutant protein. Also, under aerobic conditions, the rates are somewhat reduced with the Glu370Asp and Arg94Gly mutant dehydrogenases. These latter rates could not be analyzed by simple pseudo-first-

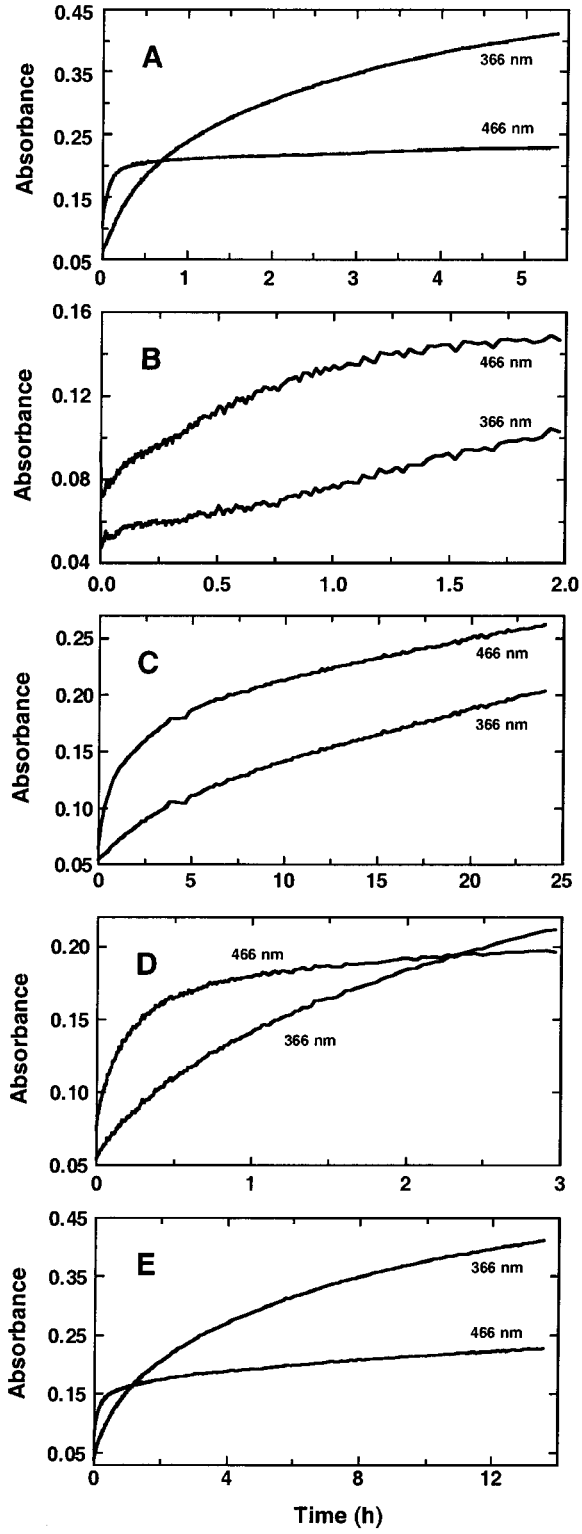


FIGURE 2: Effect of mutations in glutaryl-CoA dehydrogenase and anaerobiosis on increases in absorbance at 366 and 466 nm. The reactions were initiated by addition of 50 μM 4-nitrobutyryl-CoA to 5 μM enzyme, and the absorbance at 366 and 466 nm was monitored. Reactions were conducted in 50 mM potassium phosphate, pH 7.6, at 5 °C. The experiments show the reactions of 4-nitrobutyryl-CoA with (A) wild-type glutaryl-CoA dehydrogenase under aerobic conditions, (B) wild-type glutaryl-CoA dehydrogenase under anaerobic conditions, (C) E370Q glutaryl-CoA dehydrogenase under aerobic conditions, (D) E370D glutaryl-CoA dehydrogenase under aerobic conditions, and (E) R94G glutaryl-CoA dehydrogenase under aerobic conditions. Note the differences on the time and absorbance axes among the five experiments.

Table 2: Extent of Reduction of Flavin in Glutaryl-CoA Dehydrogenase with Glutaryl-CoA and Alternate Substrates under Anaerobic Conditions[a]

substrate	extent of reduction (%) at [S]/[E] ratio[b] of 1:1	10:1
glutaryl-CoA[c]	25.0	63.0
methyl-glutaryl-CoA	10.0	41.9
5-hexenoyl-CoA	6.6	31.1
hexanoyl-CoA	4.4	19.4
glutaramyl-CoA	3.2	20.7
pentanoyl-CoA	1.8	12.6

[a] The data were obtained in spectrophotometric titrations of wild-type glutaryl-CoA dehydrogenase with glutaryl-CoA and alternate substrates under anaerobic conditions in 50 mM phosphate, pH 7.6, containing 10% glycerol at 25 °C. [b] [S]/[E] = moles of substrate per mole of flavin. [c] Data from Dwyer et al. (*2*).

order equations since several (parallel and consecutive) reactions, enzymatic and nonenzymatic, are occurring which are pH-dependent (see Figure 5, below). Nonetheless, increases in absorbance at 366 and 466 nm in the absence of ETF, or another appropriate electron acceptor, are O_2-dependent. These absorbance changes also require the usual catalytic pathway of GCD for the reductive half-reaction of the flavin because the rates of change are significantly decreased when Glu370 in the wild-type dehydrogenase is substituted by glutamine.

To compare the kinetic parameters obtained in Table 1, and the significantly decreased capacity of 4-nitrobutyryl-CoA to reduce the flavin, the dehydrogenase was titrated with the other alternate substrates under anaerobic conditions, and the extent of flavin reduction by glutaryl-CoA and alternate substrates is shown in Table 2. Two values are listed in the table: the extent of reduction when substrate was equimolar with flavin, and the extent of reduction when substrate was in a 10-fold molar excess over flavin. Total reduction is based on reduction with $Na_2S_2O_4$ (*2*). These titrations reflect two equilibria: the equilibrium of substrate binding and the internal equilibrium involved in flavin reduction, which is dependent on the potentials of the flavin and acyl-CoA redox couples.

$$\text{glutaryl-CoA} + E_{ox} \overset{K_1}{\rightleftharpoons} [\text{glutaryl-CoA}{-}E_{ox}] \overset{K_2}{\rightleftharpoons} [\text{glutaconyl-CoA}{-}E_{2e}^{-}]$$

The turnover of GCD with these acyl-CoA thioesters is not directly reflected by the capacity of these acyl-CoAs to reduce the protein in the reductive half-reaction, but the two equilibria complicate a straightforward analysis. However, the data appear to fall into three groups. Hexanoyl-CoA and 5-hexenoyl-CoA support a high turnover, but the extent of reduction in the half-reaction is comparatively low. Both substrates exhibit very high K_m values, and the extent of reduction may be limited by substrate binding. Methylglutaryl-CoA and pentanoyl-CoA yield similar intermediate turnover numbers, and both have relatively low K_m values, but the extents of reduction are very different. It is possible that the methyl ester could hydrogen-bond with Arg94, but it is not clear why the two substrates should exhibit similar steady-state kinetic constants, yet radically different extents of reduction in the anaerobic half-reaction. Finally,

Table 3: Oxidase Activity of Human Glutaryl-CoA Dehydrogenase

substrate	oxidase activity[a] (s^{-1})	oxidase/dehydrogenase ratio (%)
glutaryl-CoA	0.023 ± 0.012	0.18
4-nitrobutyryl-CoA	0.033 ± 0.002	13.7

[a] Assayed spectrophotometrically with 35 μM glutaryl-CoA and 103 μM 4-nitrobutyryl-CoA in 10 mM Tris, pH 8.0 at 25 °C, with the Amplex red reagent. The specific activity is expressed as percent turnover.

glutaramyl-CoA would presumably form one fewer hydrogen bond with Arg94. In any case, it is clear that flavin reduction by 4-nitrobutyryl-CoA is quite unfavorable compared to the other alternate substrates. The unfavorable reduction of flavin by 4-nitrobutyryl-CoA does not appear to be based on the binding equilibrium since the K_d (Figure 1) and the K_m (Table 1) are comparable to those of other alternate substrates. The kinetic data of the reduced rate of oxidation of 4-nitrobutyryl-CoA reflect an effect on the redox potential(s) or transition state because there is a large change in $\Delta\Delta G^{\ddagger}$ (*27*).

Table 3 shows the oxidase activity of human GCD with glutaryl-CoA and 4-nitrobutyryl-CoA as substrates. The oxidase activity is similar with both substrates when assayed with the Amplex red reagent, and the two oxidase assays yield almost identical results using 4-nitrobutyryl-CoA as substrate. However, if one compares the ratio of oxidase to dehydrogenase activities, the ratio is about 75-fold greater with 4-nitrobutyryl-CoA than glutaryl-CoA. Medium-chain acyl-CoA dehydrogenase also exhibits acyl-CoA oxidase activity, and this activity is somewhat substrate-dependent (*28*, *29*).

Chemical Characterization of the Product of 4-Nitrobutyryl-CoA Oxidation. Owing to the unusual absorption of the product of 4-nitrobutyryl-CoA oxidation, and since this analogue was originally thought not to be a substrate of GCD (*5*), the product was characterized by chemical and spectral methods. In the experiment shown in Figure 3, 4-nitrobutyryl-CoA (50 μM) was incubated aerobically with wild-type GCD until the absorbance at 366 nm reached a maximum. In this experiment, the conversion of substrate to the 366 nm absorbing product was about 40% complete. The reaction mixture was then chilled to 5 °C and filtered through a Centricon YM-30 centrifugal filter. Figure 3A shows the spectrum of GCD obtained after the removal of a majority of the chromophore by centrifugal filtration. Using the estimated molar absorptivity at 366 nm for the nitronate, the concentration of nitronate in the filtrate could be calculated and, by difference, the amount of the nitro analogue remaining bound to the enzyme. The recovery of 366 nm absorbing material in the filtrate was almost quantitative. After the filtered enzyme was washed twice with buffer by centrifugal filtration, the 466 nm absorption remains with the enzyme, suggesting that the ligand is tightly bound, and by difference the amount remaining bound is nearly stoichiometric with enzyme sites. This experiment demonstrates that the increase in absorbance at 366 nm is due to a product released into solution while the 466 nm absorbance is characteristic of an enzyme-bound species. The same soluble chromophore is formed when $FcPF_6$ was used as the electron acceptor (see below).

Figure 3B shows the spectrum of the 366 nm chromophore obtained after the removal of the enzyme by the Centricon membrane filter. The absorbance at 366 nm is lost when the solution is adjusted to pH 3, and quantitatively restored when the pH is adjusted back to 7.8. For comparison, the spectrum of 4-nitrobutyryl-CoA is also included (Figure 3B, spectrum

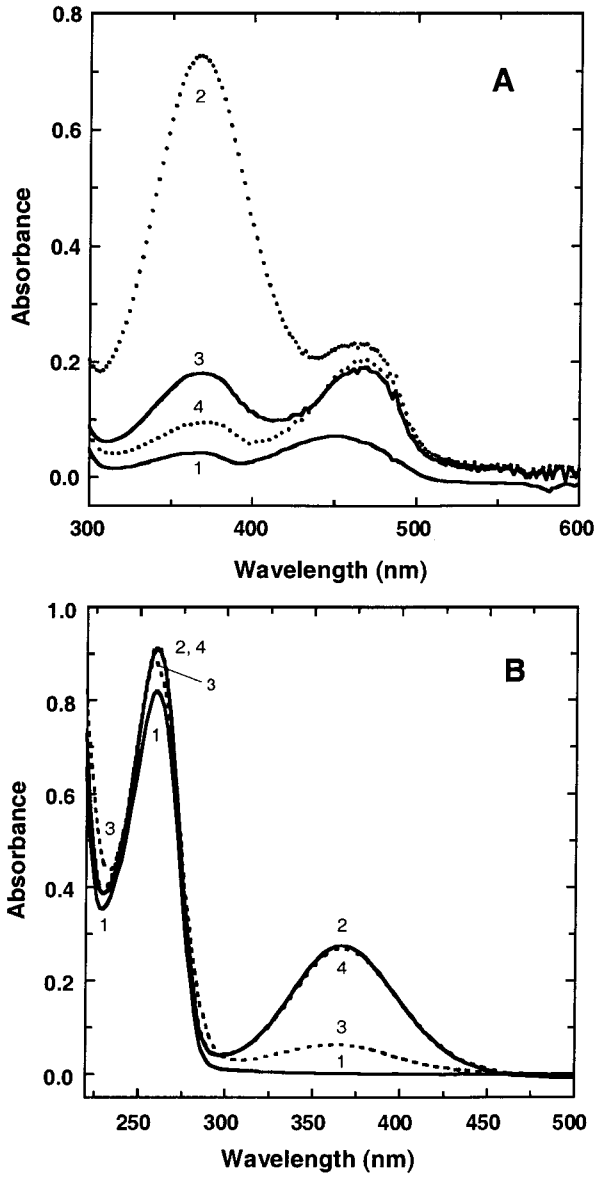


FIGURE 3: Spectra and localization of products of 4-nitrobutyryl-CoA generated by enzymatic oxidation. Panel A shows (1) the free enzyme, 5 μM, in 50 mM potassium phosphate, pH 7.6, 25 °C; (2) the enzyme 30 min after addition of 45 μM 4-nitrobutyryl-CoA under aerobic conditions; (3) enzyme after centrifugal filtration to 50 μL and dilution with the buffer to the original volume, 1 mL; and (4) the spectrum of the enzyme after a second centrifugal filtration to 50 μL and dilution with the buffer to 1 mL; by difference, about 3.0 μM of the ligand remains bound to the enzyme. The estimate of ligand remaining bound to the enzyme was based on the original concentration of nitrobutyryl-CoA and the concentration of the 366 nm chromophore using $\epsilon_{366nm} = 14.5\ mM^{-1}\ cm^{-1}$. Panel B shows (1) 50 μM 4-nitrobutyryl-CoA in 50 mM potassium phosphate, pH 7.6, 25 °C; (2) the filtrate after removal of the enzyme by centrifugal filtration; the 366 nm absorbing material was generated by incubation with glutaryl-CoA dehydrogenase under aerobic conditions as described in panel A; (3) acidification of the filtrate in spectrum 2 to pH 3.12 with 1 M phosphoric acid (after correction for dilution); and (4) the spectrum of the acidified filtrate in spectrum 3 after adjusting the pH to 7.80 with 1 M potassium hydroxide (corrected for dilution). Note that spectra 2 and 4 are essentially identical.

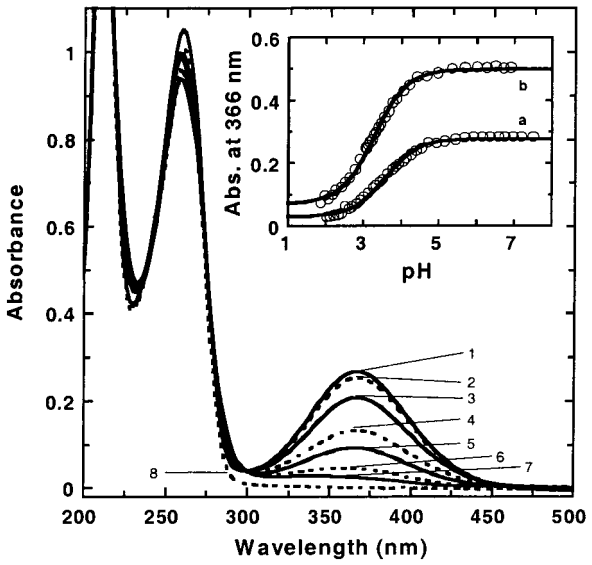


FIGURE 4: pH titration of the free 366 nm absorbing acyl-CoA. The 366 nm absorbing material was generated by the activity of wild-type glutaryl-CoA dehydrogenase on 4-nitrobutyryl-CoA under aerobic conditions in 50 mM potassium phosphate buffer, pH 7.6, 25 °C. After removal of the enzyme by centrifugal filtration, the enzyme was titrated with 1 M phosphoric acid. The final pH values are (1) 7.6, (2) 6.1, (3) 5.9, (4) 5.6, (5) 4.9, (6) 2.9, and (7) 2.4. For comparison, the spectrum of 4-nitrobutyryl-CoA, 50 μM at pH 7.6 (8), is also included. The inset shows the results of spectrophotometric titrations with citric acid monitored at 366 nm. The 366 nm absorbing compound was generated with wild-type glutaryl-CoA dehydrogenase with (a) oxygen and (b) 200 μM $FcPF_6$ as the electron acceptors. Individual experimental data points are shown, and the curve was obtained by fitting the data to a single ionization equation as described by Dwyer et al. (*2*).

1). However, spectrum 3 is not identical to spectrum 1 suggesting that, upon acidification, the nonchromophoric enoyl-CoA, 4-nitro-but-2-enoyl-CoA, is produced. The absorption spectrum of the product with 366 nm absorbance is closely related to those of conjugated 2,4-dienoyl-CoA thioesters (*30, 31*), suggesting that the 366 nm chromophore is 4-nitronate-but-2-enoyl-CoA. The absorbance maximum is red-shifted into the visible region due to (a) extended conjugation and (b) charge delocalization with several possible canonical structures.

To further characterize the 366 nm absorbing product, the product was generated with oxygen as the electron acceptor as above, the enzyme was removed by centrifugal filtration, and then the product (filtrate) was titrated with 1 M H_3PO_4 while monitoring the absorption spectrum between 200 and 500 nm (Figure 4). The presence of isosbestic points at 299, 275, and 245 nm during the titration indicates the presence of only two absorbing species under equilibrium conditions. For comparison, the spectrum of 4-nitrobutyryl-CoA in 50 mM potassium phosphate, pH 7.6, is included. It is obvious that the substrate is not part of the equilibrium mixture. The maximum absorbance change occurred at 366 nm. The inset of Figure 4 shows spectrophotometric titrations of the product monitored at 366 nm. All spectra were corrected for dilution (less than 10%) due to addition of citric acid. Since the two absorbing species are in equilibrium, the titration data can be evaluated to obtain their pK_a by analyzing the absorbance data at 366 nm. The chromophoric product generated aerobically (curve a) exhibits a p$K_a = 3.60 \pm 0.02$, and the 366 nm absorbing product generated with $FcPF_6$ (curve b) as the electron acceptor exhibited a p$K_a = 3.30 \pm 0.02$. The similarity of the absorption spectra and the similar pK_as suggests that the products of the oxidase and dehydrogenase reactions are identical. This product can be generated by dehydrogenase and oxidase activities of GCD, both of which are affected by mutations at Glu370, as is the dehydrogenase activity with glutaryl-CoA (*2*). Incubation of the sodium salt of 4-nitrobutyric acid over a range of pHs while monitoring the absorbance at 230 nm, until equilibrium was reached, indicated that it has a pK_a of 7.1 (data not shown); however, as expected, equilibration is very slow, occurring over a period of at least 12 h. We attribute this titrable group to the protonation−deprotonation of the nitroalkane−alkylnitronate equilibrium. The pK_a of the γ-proton of 4-nitro-but-2-enoyl-CoA, the presumed product, is about 3.5. This decrease in pK_a is consistent with the assignment of the 366 nm absorbing product as the conjugated nitronate. For example, the pK_a of 1-nitrobutane is 8.6 (*32*) while that of nitrobut-2-ene is 5.2 (*33*). The reduction of the pK_a of the model compound and the enoyl-CoA is about 3.5−4.0 pH units when the conjugated double bond is introduced. This pK_a does not reflect the protonation/deprotonation of the oxygens in the "nitronate" group to form the nitronic acid, i.e., 4-*aci*-nitro-but-2-enoyl-CoA. (The nomenclature of nitro compounds is given in ref *34*.) The conclusion that the 366 nm absorbing product is the nitronate is based on the demonstration that protonation of the chromophoric product results in the loss of absorbance at 366 nm that is attributed to loss of the extended conjugation, forming 4-nitro-but-2-enoyl-CoA. Moreover, the 366 nm chromophore is regained quantitatively upon adjusting the pH back to pH 7.8 (Figure 3B). It is known that nitronic acids are ~2 pK_a units more acidic than the corresponding nitroalkene, indicating that the concentration of the nitronic acid form would be very low and well below experimental errors (*33*). Finally, the conclusion that the product at low pH is 4-nitro-but-2-enoyl-CoA, rather than the corresponding nitronic acid, is supported by the fact that 1-nitroalkane is thermodynamically more stable than the corresponding nitronic acid (*34*).

NMR and Mass Spectrometry of the 366 nm Absorbing Product. The 366 nm absorbing product was further analyzed by ^{1}H NMR. We generated the product in the absence of an exogenous electron acceptor to avoid possible interference in analyses. This strategy was adopted because it was not possible to purify the product owing to several possible side reactions of the unstable 4-nitronate-but-2-enoyl-CoA with buffer ions, hydroxide ions, O_2 or H_2O_2, and cyclization and hydrolysis (*34*). 4-Nitrobutyryl-CoA was characterized by ^{1}H NMR spectroscopy, with all chemical shifts in agreement with literature for free coenzyme A and acyl-CoAs (*35*−*37*), and the 4-nitrobutyryl signals at 2.66 (H2‴), 2.19 (H3‴), and 4.46 ppm (H4‴), confirmed by a COSY spectrum. Analysis of the 366 nm absorbing product indicates formation of a double bond. The key signals are protons at 6.16 and 7.47 ppm, shown by COSY to be coupled. The shifts are indicative of a C2−C3 double bond, consistent with the activity of the dehydrogenase. The coupling constant, $J =$ 15.6 Hz, indicates the double bond is *trans*. No signal for the proton at the γ-carbon was observed since the NMR samples at pH 8 were ionized (nitronate form), making the single proton on C-4 of 4-nitronate-but-2-enoyl-CoA solvent-

Table 4: Mass Spectral Data of the Products of 4-Nitrobutyryl-CoA Oxidation in the Presence and Absence of Ferrocenium Hexafluorophosphate

positive ion mode		negative ion mode		
m/z[a]	intensity (cps × 10^{-5})[b]	*m/z*	intensity (cps × 10^{-5})	compound
		Electron Acceptor = $FcPF_6$		
881.3	1.41	879.2	1.38	4-nitronate-but-2-enoyl-CoA
840.4	0.37	838.6	0.24	3-hydroxy-propanoyl-CoA
767.6	0.62	765.8	3.30	oxidized CoA
		Electron Acceptor = Oxygen		
881.4	1.26	879.6	1.38	4-nitronate-but-2-enoyl-CoA
854.5	0.86	852.4	0.84	4-hydroxy-butyryl-CoA
840.4	0.92	838.4	1.13	3-hydroxy-propanoyl-CoA
838.5	1.41	836.5	1.74	2-keto-propanoyl-CoA[c]
768.3	0.83	nd[d]	nd	reduced CoA
767.5	0.79	765.7	1.83	oxidized CoA

[a] *m/z* of acid forms (higher molecular weight salts not given). [b] cps is counts per second. [c] Also equivalent to 3-hydroxy-prop-2-enoyl-CoA. [d] Not detected.

exchangeable owing to the pK_a of about 3.5. Olefinic signals, which we attribute to the chromophoric product (since it must have extended conjugation not present in the starting material), are lost after 1−2 days, indicating it is unstable in solution. A second product evidently corresponds to truncation of the acyl group to $CH_2(OH)-CH_2-C(=O)-SCoA$, with proton signals at 2.67 and 3.33 ppm, respectively. This product could be accounted for by a reverse Michael addition with nucleophilic attack at the β-carbon of the 4-nitronate-but-2-enoyl-CoA, giving an acrylate ester (acrylyl-CoA), followed by Michael addition of water to give 3-hydroxy-propanoyl-CoA. 3-Hydroxy-propanoyl-CoA may also arise by hydration of acrylyl-CoA through the enoyl-CoA hydratase activity of GCD (*38*). This assumes that acrylyl-CoA could be formed by elimination of methenyl nitronate as described above. This occurs only during the relatively slow generation of 4-nitronate-but-2-enoyl-CoA with oxygen as the electron acceptor. 3-Hydroxy-propanoyl-CoA in the starting material is significantly less than that detected among the products of the enzymatic oxidation with oxygen as the electron acceptor. Therefore, the majority of 3-hydroxy-propanoyl-CoA is more likely derived from 4-nitronate-but-2-enoyl-CoA by elimination of the methenyl nitronate and hydration of the double bond.

To complement the NMR results, the product of 4-nitrobutyryl-CoA oxidation was analyzed by mass spectrometry. When the product was generated in the presence of $FcPF_6$, the sole product exhibited ions at *m/z* 881.3 ($[M+2H]^+$) and 879.2 ($[M]^-$) (Table 4). Since 4-nitronate-but-2-enoyl-CoA is the chromophoric anion with unit negative charge ($C_{25}H_{38}N_8O_{19}P_3S$; calculated average mass = 879.6), the *m/z* value in the negative ion mode does indicate the exact nonisotopic molecular mass (879.2). This result is in agreement with the *m/z* value in the positive ion mode wherein two protons are added to the chromophore to obtain a unit positive charge. One other species, with ions at *m/z* 840.4 ($[M+H]^+$) or 838.6 2 ($[M-H]^-$), was also observed in the synthetic substrate. In addition to these species, reduced and oxidized forms of CoASH were also detected. Consistent with the results of NMR spectroscopy, several products were detected by the mass spectrum when the chromophore was generated with molecular oxygen as the electron acceptor, the primary product being the same as above ($[M+2H]^+$, *m/z* 881.4; and $[M]^-$, *m/z* 879.6) (Table 4). The possible identities of the side products are indicated in Table 4. All of these can be explained in terms of the chemistry of nitronate compounds, which are somewhat unstable even in slightly alkaline aqueous media (*34*).

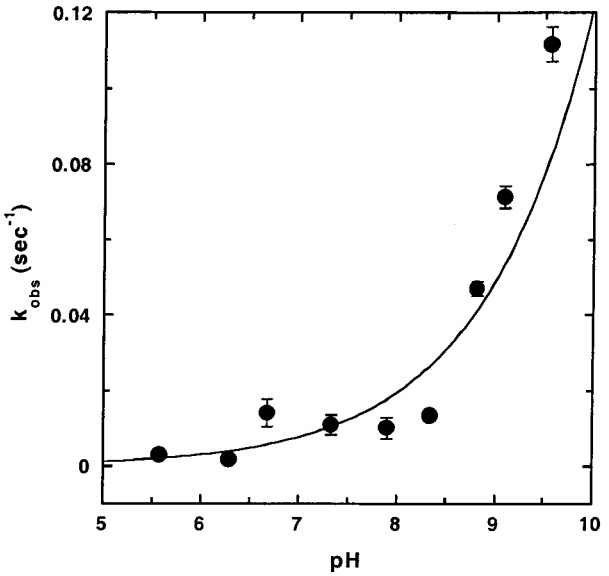


FIGURE 5: pH dependence of the pseudo-first-order rate constant, k_{obs}, for the formation of 4-nitronate-but-2-enoyl-CoA. The reaction was monitored at 366 nm in the presence of 5 μM wild-type glutaryl-CoA dehydrogenase and 52 μM 4-nitrobutyryl-CoA at 25 °C in various buffers. The buffers, 20 mM adjusted to a constant ionic strength of 200 mM with NaCl, and their pH were as follows: 2-(*N*-morpholino)ethanesulfonate-K^+ (pH 5.6), Bis-Tris-Cl (pH 6.3 and 6.7), Tris-HCl (pH 7.3 and 7.9), 2-amino-2-methyl-1,3-propanediol-HCl (pH 8.3, 8.8, 9.1, and 9.6). The error bars are indicated when larger than the size of the symbols.

Enzyme-Catalyzed and Chemical Formation of 4-Nitronate-but-2-enoyl-CoA. Chemical and spectral data show that the product of 4-nitrobutyryl-CoA oxidation that exhibits absorbance at 366 nm is the nitronate. Introduction of the *trans* double bond at C-2 is enzyme-catalyzed. However, to form the nitronate, the 2-enoyl-CoA must be deprotonated at the γ-carbon. It seems unlikely that this deprotonation occurs in the low dielectric environment of the active site without a second general base catalyst. However, from the crystal structure (*23*), it is not possible to identify a second base in the active site that would catalyze the deprotonation. Gomes et al. showed that the α-proton abstracted by the catalytic base of a bacterial GCD does not exchange with solvent (*4*). If the human enzyme functions similarly, it is unlikely that the α-proton dissociates from Glu370, and that the Glu370 base abstracts the γ-proton in the binary complex. However, it is possible that the 2-enoyl-CoA dissociates and then binds a second time so that Glu370 would have access to the γ-proton, which would have a lower pK_a.

The pH dependence of nitronate formation under pseudo-first-order conditions shows that the rate of nitronate formation increases as a function of pH (Figure 5). Nitronate formation is, at least in part, enzymatic, and the nitronate is unstable, especially at high pH (*34*); therefore, only the pseudo-first-order rate constant, k_{obs}, was determined at each pH. The rate of γ-deprotonation to form the nitronate was determined at 25 °C from the increase in absorbance at 366 nm. Reactions were conducted in the buffers indicated in

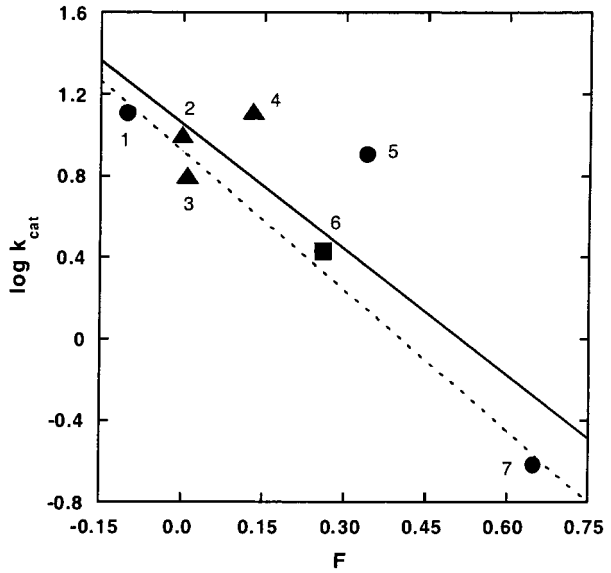


FIGURE 6: Correlation of log(k_{cat}) with the field parameter, *F*, that describes the 'through-bond' inductive and 'through-space' field effects of the γ substituents without a contribution from resonance effects. The γ substituents are (1) carboxylate, (2) ethyl, (3) methyl, (4) vinyl, (5) methyl ester, (6) amide, and (7) nitro. The substituents also differ in their ability to act as hydrogen bond donor or acceptor. The symbols indicate: (●) hydrogen bond acceptor but not a donor, (▲) neither hydrogen bond donor nor acceptor, (■) either a hydrogen bond donor or an acceptor.

the legend of Figure 5, and the ionic strength was maintained at 200 mM by the addition of NaCl. The data were fit to the equation (*39*):

$$k_{obs} = k_1^{OH^-} a_{OH^-} + k_1^{B^-}[B^-]$$

where $k_1^{OH^-}$ and $k_1^{B^-}$ are the hydroxide- and buffer-driven second-order rate constants for the ionization of 4-nitro-but-2-enoyl-CoA, a_{OH^-} is the activity of hydroxide ions, and [B^-] is the buffer concentration determined from the pK_a values of the buffers. Analysis of the data using the dual-parameter equation using Sigma Plot (SPSS Science) yields $k_1^{OH^-}$ = 2514 ± 535 M^{-1} s^{-1} and $k_1^{B^-}$ = 2.1 ± 1.6 M^{-1} s^{-1} (r^2 = 0.927, standard error = 0.0119) for the ionization of 4-nitro-but-2-enoyl-CoA. Thus, the reaction appears to be driven chiefly by hydroxide ions. This result and the fact that the pH optimum of the dehydrogenase reaction is 8.2 support the conclusion that γ-deprotonation of the 2-enoyl-CoA occurs in solution.

QSAR Analysis of Glutaryl-CoA Dehydrogenase Catalysis. The data in Table 1 suggest at least a qualitative correlation of k_{cat} with the inductive effects of γ-substituents of butyryl-CoA. These substituents can be classified as either electron-donating (+I) or electron-withdrawing (−I) (*40*). The data in Table 1 show a decrease in k_{cat} as the substituents become more electron-withdrawing. To address this issue in quantitative terms, we used QSAR to elucidate aspects of the reaction mechanism (*22*). The effects of substituents on reactivity can be divided into three major types: field, resonance, and steric (*40*). In aliphatic systems, resonance effects are unimportant, so that substituent effects are transmitted mainly by field (field-inductive) effects. Field-inductive effects, which include both 'through-bond' and 'through-space' effects, increase when electronegative substituents are present (*41*). Relatively nonspecific interactions such as the partition coefficient (*P*), hydrophobicity parameter (π), and molar refractivity (MR) are not likely to play a major role in enzyme−substrate reactions (*22*). Seven γ-substituents of butyryl-CoA were studied (Table 1). The two reaction centers are the α-carbon (for proton abstraction), the 'core' being the two methylene groups at the β and γ positions, and the β-carbon (for hydride transfer), the 'core' being the methylene group at the γ position. It is reasonable that any inductive effect would be more pronounced at the β-carbon due to proximity.

The steady-state kinetic constants shown in Table 1 were analyzed with the following substituent parameters: resonance (*R*), partition coefficient (*P*), and hydrophobicity parameter (π), molar refractivity (MR), steric effects (E_s), the van der Waals' volume factor (V_w), and field-inductive effects (*F*). In aliphatic systems, since there is no π electronic system or aromaticity, the omission of the resonance parameter is justified. No correlation between log(K_m), log(1/K_m), or log(k_{cat}/K_m) and any of the substituent parameters was found using single-parameter linear regression, and no better correlation was obtained using dual-parameter analysis.

The linear response when log(k_{cat}) was plotted as a function of the field parameter, *F*, suggests that the underlying mechanism for the oxidation of these substrates is the same (Figure 6). Good correlation was obtained using all the experimental data points.

$$\log(k_{cat}) = 1.057(\pm 0.167) - 2.062(\pm 0.557)F$$

$$n = 7,\ r^2 = 0.733,\ s = 0.350$$

The correlation improved significantly when data points 4 and 5 in Figure 6 were removed from the analysis, yielding

$$\log(k_{cat}) = 0.920(\pm 0.054) - 2.295(\pm 0.170)F$$

$$n = 5,\ r^2 = 0.984,\ s = 0.103$$

The reason for the deviation of the 5-hexenoyl-CoA from the fit is not clear because we do not yet have the three-dimensional structure of the enzyme with a bound acyl-CoA ligand. It is possible that the data with the methyl ester deviate because the methyl group can bend out of the way in the active site and the methoxy oxygen could still function as a hydrogen bond acceptor from Arg94 (*3*, *23*). Explanations based on accommodation of substrate in the active site are not related to the steric factors, E_s and V_w, because these parameters refer to access to the reaction center. In our case, there is no steric hindrance by substituents at the reaction centers. However, orientation and positioning of the α-proton and β-hydride may change due to steric constraints in the active site that would be reflected in both k_{cat} and K_m for these substrates (*42*). Since resonance does not play a role in the aliphatic system, the effects of substituents are due to 'through-bond' (true inductive or electronegative) or 'through-space' (field) effects (*41*). In the case of an enzyme-catalyzed reaction, it is difficult to access the relative contributions of these effects though predominance of 'through-space' (field) effects has been suggested (*22*, *41*). However, owing to the large residuals and limited range of substrates that could be studied here, one cannot rule out the possibility of other contributing factors to the QSAR.

The reaction constant, ρ, is a measure of the sensitivity of a reaction to the effects of electronic perturbation and the ability of the 'core' to transmit electronic effects (*41*). The negative slope, ρ, in Figure 6 indicates a large electron demand at the reaction center, implying that the reduction of electron-deficient flavin is the dominating factor (*22, 40, 41*). However, the influence of substituents on either the rate of proton abstraction from the α-carbon or the pK_a of α-protons cannot be completely ruled out. In the oxidation of substrates by GCD, if the rate-determining step is one of the chemical steps, the QSAR result would suggest that the hydride transfer from β-C to N-5 of the flavin could be it (*43*). In the absence of other experimental evidence, the contribution of physical processes of adsorption and/or desorption of either substrate or products toward the rate-determining step cannot be ruled out since QSAR accounts for the chemical steps only.

SUMMARY

The contribution of electrostatics to rate enhancement in enzyme catalysis has received increased experimental and theoretical support over the last several years. Electrostatics is critical in the low dielectric constant of the enzyme active site with the resulting high field strength, and because of substrate polarization generated by charge separation (*44*). These factors are well illustrated in the catalytic pathways of the acyl-CoA dehydrogenases. Charge migrates from a glutamate general base catalyst to a substrate α-carbanion and then, with hydride transfer, to an anionic dihydroflavin (*8*). Among members of the acyl-CoA dehydrogenase family, electrostatics acquire additional significance in the reaction catalyzed by GCD. GCD is the only acyl-CoA dehydrogenase to catalyze the oxidation of a charged acyl group. The charge on the acyl group increases from −1 to 0 during the course of the oxidative decarboxylation when the flavin cycles from an anionic dihydroquinone to an anionic semiquinone and then to the oxidized state (*2*). The catalytic pathway also involves a crotonyl-CoA anion that is reprotonated by a general acid catalyst, apparently the conjugate acid of the glutamate base, Glu370 (*4*). GCD is the only member of the acyl-CoA dehydrogenase family that contains a basic amino acid, Arg94, in the active site (*3, 23*). The γ-carboxylate of glutaryl-CoA is within hydrogen-bonding distance of the guanidinium group of Arg94 when glutaryl-CoA is modeled into the active site. The model was constructed such that the thioester oxygen of glutaryl-CoA was hydrogen-bonded by the backbone NH of Glu370 and the 2′-hydroxyl of FAD as in other acyl-CoA dehydrogenases (*23*−*25*). In addition to positioning the substrate for dehydrogenation, Arg94 also functions to neutralize negative charge in the active site as an electrostatic catalyst (*3*).

Catalysis by the acyl-CoA dehydrogenase family involves several common mechanistic aspects that may be altered in GCD by substitutions at the γ position for the carboxylate in glutaryl-CoA. First, the pK_a of the α-proton of the substrate is reduced to facilitate abstraction by general base catalysis. The reduction of that pK_a depends on polarization of the substrate by hydrogen-bonding the thioester oxygen (*10, 14, 23*−*26, 43*). Second, a transition state with partial positive charge on the β-carbon must be stabilized to permit hydride transfer to the flavin (*14, 43*). This stabilization depends on the same mechanism of substrate polarization via the thioester oxygen (*14*). Finally, the oxidation−reduction potential of the flavin must be positively modulated by stabilization of partial positive charge at the β-carbon on the enzyme-bound enoyl-CoA product (*14, 43, 45*−*47*). This positive modulation is required to make reduction of the flavin thermodynamically favorable and is also mediated by polarization of the thioester oxygen by hydrogen-bonding (*14, 45*−*47*). The mechanism of the reductive half-reaction of the GCD flavin appears to be essentially identical to those of the other acyl-CoA dehydrogenases (*2, 3*), and the redox potential of the GCD flavin is apparently similarly regulated (*2, 45*−*47*).

4-Nitrobutyryl-CoA is a very poor substrate of GCD despite its structural and electronic similarity to glutaryl-CoA. Differences in the free energy of binding, $\Delta\Delta G_b$ (*27*), do not explain the low turnover of the enzyme with the nitrobutyryl-CoA analogue. Regarding the common facets of acyl-CoA dehydrogenase catalysis enumerated above, the turnover of GCD with 4-nitrobutyryl-CoA and the other alternate substrates is strongly influenced by field-inductive effects at the β-carbon that are expected to increase the redox potential of the substrate/product couple. In the extreme case, the electron-withdrawing inductive effect of the nitro group makes hydride transfer to the flavin less favorable. The nitro substituent increases the potential of this substrate/product couple, and the partial positive charge that must develop at the β-carbon in the transition state is not favored (*14*). It is not favored due to the formal positive charge on the nitrogen of the zwitterionic nitro group with its oxygen hydrogen-bonded to Arg94. Finally, if partial positive charge at the β-carbon cannot be accommodated due to the positively charged nitrogen, the potential of the flavin cannot be positively modulated to make flavin reduction by the substrate favorable. These considerations explain the decreased k_{cat}, and the inability of 4-nitrobutyryl-CoA to reduce the dehydrogenase flavin in spectrophotometric titrations. There is no reason to expect that the thioester carbonyl oxygen of 4-nitrobutyryl-CoA would not be adequately accommodated, and serve as a hydrogen bond acceptor as an oxyanion following deprotonation. Formation of the 466 nm absorbing species shows a strong dependence on the presence of the catalytic base, Glu370. Further, this species can be formed in a 1:1 stoichiometry with sites under anaerobic conditions. Its formation is slowed in the Glu370Asp mutant which moves the carboxylate of Glu370 about 1 Å further from the *pro-R* α-hydrogen of the substrate (*2*). Similarly, mutation of Arg94 to Gly slows formation of the species. Arg94 functions by binding the γ-carboxylate of glutaryl-CoA, positioning the substrate in the active site, and participating as an electrostatic catalyst (*3*). Also, the species is rather tightly bound to the active site. Finally, addition of the 4-nitronate-but-2-enoyl-CoA to the free enzyme does not perturb the flavin spectrum. Thus, it is reasonable to propose that the 466 nm absorbing species results from perturbation of the flavin spectrum by the delocalized anion (enolate) of 4-nitrobutyryl-CoA. The nitro group is also expected to have some effect on the acidity of the α-proton. The effect could be propagated through the alkyl chain, decreasing by about 30% at each methylene (*48*).

QSAR studies with log(k_{cat}) are not unprecedented since in an enzyme-catalyzed reaction, there are two free energy functions, ΔG_{bind} and $\Delta G^{\ddagger}_{cat}$. In most enzymatic reactions, ΔG_{bind} is negative, and the maximum rate when substrate is

saturating is given approximately by k_{cat}, which depends only on $\Delta G^{\ddagger}_{cat}$ (*22*, *49*). The present QSAR analysis suggests the following: (a) the mechanism of substrate oxidation by GCD may be the same with all the substrates studied, and (b) electronic effects in the active site on developing positive charge on the β-carbon of the substrate may dominate flavin reduction. It remains to be established that a chemical step is rate-determining in the steady state.

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