Spectral and Electrochemical Properties of Glutaryl-CoA Dehydrogenase from *Paracoccus denitrificans*†

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ABSTRACT: Studies of the spectral (UV/vis and resonance Raman) and electrochemical properties of the FAD-containing enzyme glutaryl-CoA dehydrogenase (GCD) from *Paracoccus denitrificans* reveal that the properties of the oxidized enzyme (GCD_{ox}) appear to be invariant from those properties known for other acyl-CoA dehydrogenases such as mammalian general acyl-CoA dehydrogenase (GACD) and butyryl-CoA dehydrogenase (BCD) from *Megasphaera elsdenii*. However, when either free or complexed GCD is reduced, its spectral and electrochemical behavior differs from that of both GACD and BCD. Free GCD does not stabilize any form of one-electron-reduced GCD, but when GCD is complexed to its inhibitor, acetoacetyl-CoA, the enzyme stabilizes 20% of the blue neutral radical form of FAD ($FADH^{\bullet}$) upon reduction. Like GACD, when crotonyl-CoA- (CCoA) bound GCD is reduced, the red anionic form of FAD radical ($FAD^{\bullet-}$) is stabilized, and excess reduction equivalents are necessary to effect full reduction of the complex. A comproportionation reaction is proposed between fully reduced crotonyl-CoA-bound GCD (GCD_{2e}–CCoA) and GCD_{ox}–CCoA to partially explain the stabilization of GCD-bound $FAD^{\bullet-}$ by CCoA. When GCD is reduced by its optimal substrate, glutaryl-CoA, a two-electron reduction is observed with concomitant formation of a long-wavelength charge-transfer band. It is proposed that the ETF specific for GCD abstracts one electron from this charge-transfer species and this is followed by the decarboxylation of the oxidized substrate. At pH 6.4, potential values measured for free GCD and GCD bound to acetoacetyl-CoA are −0.085 and −0.129 V, respectively. Experimental evidence is given for a positive shift in the reduction potential of GCD when the enzyme is bound to a 1:1 mixture of butyryl-CoA and CCoA. However, significant GCD hydratase activity is observed, preventing quantitation of the potential shift.

Glutaryl-CoA dehydrogenase from *Paracoccus denitrificans* catalyzes the oxidative decarboxylation of glutaryl-CoA to crotonyl-CoA in its role as an enzyme in the pathway of L-lysine, L-hydroxylysine, and L-tryptophan metabolism. As with other acyl-CoA dehydrogenases, GCD[1] donates its reaction product into the well-known pathway of β-oxidation and its electrons to ETF, where they continue through a series of enzymes into the electron-transport chain. Characterization of mammalian GCD is difficult as it is present only in small amounts in mitochondria (Lenich & Goodman, 1986) in contrast to dehydrogenases such as GACD whose relative abundance in the mitochondrial matrix have facilitated the study of their spectral and oxidation–reduction properties (Thorpe et al., 1979; Mizzer & Thorpe, 1981; Lenn, 1989; Finocchiaro et al., 1987; Gustafson et al., 1986) and their reaction mechanisms (Schopfer et al., 1988). However, GCD can be obtained in large amounts from *Paracoccus denitrificans* (Husain & Steenkamp, 1985), a bacterium that possesses many striking similarities in its electron-transport and oxygen-transport systems to those of mitochondria (John & Whatley, 1975). In fact, after reduction with their respective substrates, *Paracoccus* GCD and many of the mammalian ETF-dependent dehydrogenases are able to donate reducing equivalents to either *Paracoccus* ETF or pig liver ETF (Husain & Steenkamp, 1985). Therefore, it is believed that characterization of the *Paracoccus* GCD will facilitate the study of mammalian GCD.

Preliminary spectral properties of sodium dithionite reduced *Paracoccus* GCD and acetoacetyl-CoA-reduced *Paracoccus* GCD_{ox} have been published (Husain & Steenkamp, 1985). Through the use of UV/visible spectroscopy, electrochemistry, and resonance Raman spectrometry, this work further explores the effects of the interaction of substrates, substrate analogues, inhibitors, and products with GCD. Of specific interest was the reduction of GCD by optimal and nonoptimal substrates, the determination of the electron-transfer properties of GCD, and the question as to whether the electron-transfer properties of GCD are regulated by substrate and product binding. Such regulation of enzyme redox properties by substrate and product binding have been observed with pig kidney GACD and BCD from *Megasphaera elsdenii* (Lenn, 1989; Stankovich & Soltysik, 1987). Comparison of the data obtained for GCD with those previously reported for pig kidney GACD and BCD from *M. elsdenii* highlights those properties common among the ACDs and some properties that appear to be specific for GCD.

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[1] Abbreviations: 8ClRfl, 8-chlororiboflavin; ACD, acyl-CoA dehydrogenase; BCD, butyryl-CoA dehydrogenase; BCoA, butyryl-CoA; CCoA, crotonyl-CoA; GCoA, glutaryl-CoA; E_m, midpoint potential; $\hat{E}$, conditional midpoint potential; ETF, electron-transfer flavoprotein; GACD, general acyl-CoA dehydrogenase; $GACD_{1e}$, one-electron-reduced GACD; $GACD_{2e}$, two-electron-reduced (fully) GACD; GCD, glutaryl-CoA dehydrogenase; GCD_{ox}, fully oxidized GCD; GCD_{1e}, one-electron-reduced GCD; GCD_{2e}, two-electron-reduced (fully) GCD; LFA, lumiflavin 3-acetate; MV, methylviologen; PYC, pyocyanine; RR, resonance Raman.

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Materials and Methods

Materials. The dye PYC was photochemically synthesized by the method of McIlwain (1937) using phenazine methosulfate from Aldrich as the starting material. Indigo disulfonate was obtained from MCB. Lumiflavin 3-acetate and 8ClRfl were the generous gifts of Dr. Sandro Ghisla, University of Konstanz, FRG, and Dr. J. P. Lambooy, University of Maryland, respectively. Riboflavin was purchased from Eastman Kodak. Methylviologen was purchased from British Drug House, Poole, England, and dissolved in water to a concentration of 1.000×10^{-3} M. Acetoacetyl-CoA, BCoA, CCoA, β-hydroxybutyryl-CoA, and GCoA were purchased from Sigma or Pharmacia P-L Biochemicals and were either weighed out for a particular experiment or dissolved in water and stored frozen. Sodium dithionite, potassium ferricyanide, and potassium ferrocyanide were obtained from J. T. Baker. Both 1,6-diaminohexane–agarose and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide were purchased from Sigma. Doubly glass-distilled water was used for all experiments.

Ethylene glycol from Mallincrodt or Fisher (analytical grade) was added to all buffers to a final concentration of 10% (v/v).

Synthesis of Agarose–(Aminohexyl)glutaryl-CoA. Approximately 50 mL of (aminohexyl)agarose (1,6-diaminohexane–agarose) which had been thoroughly washed with water was suspended in 30 mL of cold water and stirred. 1-Ethyl-3-[3-(dimethylamino)propyl]carbodiimide (200 μmol) was added quickly, followed by 200 μmol of GCoA dissolved in 15 mL of water. The pH of the suspension was adjusted and maintained at 5 with 1 M acetic acid. When the pH of the suspension stabilized (approximately 1 h), the suspension was left overnight at 5 °C. The resin was filtered and washed extensively with water.

Enzyme Purification. Glutaryl-CoA dehydrogenase was purified from *P. denitrificans* by a slight modification of the method of Husain and Steenkamp (1985). The dialyzed enzyme from DEAE-Sepharose was applied to the agarose–(aminohexyl)glutaryl-CoA column (1.5 × 20 cm), which had previously been equilibrated with 10 mM potassium phosphate (pH 6.5). The column was successively washed with 500 mL of the starting buffer and 500 mL of the starting buffer containing 100 mM NaCl. The enzyme was eluted with a 1-L linear gradient of 100–500 mM NaCl. Active fractions were pooled and concentrated. No loss of the flavin chromophore was observed during this step in contrast to hydroxyapatite column chromatography, which has been seen to cause substantial loss of the flavin from the enzyme. The specific activity and A_{270}/A_{435} ratio of GCD from the affinity column were comparable to those reported for the homogeneous enzyme. The enzyme was stored in a light-tight container in a −20 °C freezer.

Methods. Electrochemical experiments were performed with either the PAR 173 potentiostat and 175 coulometer or the BAS 100 electrochemical system using the spectroelectrochemical methodology described in Stankovich (1980) and Stankovich and Fox (1983). Spectra were obtained and stored on Apple 2+ or 2e interfaced Cary 210 and 219 spectrophotometers with thermostated cell compartments. Unless otherwise stated, all spectroelectrochemical studies of GCD were performed at 4 °C. In addition, great care was taken in all experiments involving GCD and dyes to keep light to a minimum.

Coulometric Titrations. Coulometric titrations were performed on free (uncomplexed) GCD in the following manner. Approximately 4 mL solutions of 10–20 μM GCD and 100 μM MV^{2+} at pH 6.4 or 6.0 were filtered into the spectroelectrochemical cell. For consistency, the following pattern was adopted: add charge, stir 10 min; wait an additional 10 min before taking each spectrum. Coulometric analyses of GCD at pH 6.4 in the presence of excess acyl-CoA derivatives were performed in a similar manner in which the analyte solution contained 10–20 μM GCD, 100 μM MV^{2+}, and either 35 μM acetoacetyl-CoA, 150 μM CCoA, or 150 μM β-hydroxybutyryl-CoA in buffer, pH 6.4. However, in the experiment containing CCoA, full reduction of the enzyme was difficult because of rapid reoxidation of GCD_{2e}. Therefore, some spectra were recorded immediately following charge addition. When noted, the experimental spectra were corrected for the interference of protein turbidity as described by Byron et al. (1987).

Potentiometric Titrations. These experiments were performed in various ways on free GCD and on GCD in the presence of acetoacetyl-CoA, CCoA, a mixture of BCoA and CCoA, and β-hydroxybutyryl-CoA with each GCD–acyl-CoA complex comprising a separate analyte solution. Potentiometric titrations were usually done in a manner similar to that of the coulometric titrations in which aliquots of charge were added to the system and allowed to stir for 5 min. The criteria for equilibrium were a <0.0003 change in absorbance units per 5 min and a <0.001 V change in cell potential per 5 min. Some alternative potential measurements (nontitration) were made and will be noted under Results.

Indicator dyes that interfere spectrally with the potentiometric analyses were titrated alone in buffer to determine their reduction potentials and to note their spectral properties as a function of measured potential such that GCD potentiometric data could be corrected. Further, it was shown by Stankovich and Soltysik (1987) that the properties of the dyes used in these experiments do not change in the presence of acyl-CoA derivatives.

For free GCD at pH 6.4, the analyte solution consisted of 10–20 μM GCD, 100 μM MV^{2+}, and 1 μM each of the indicator dyes LFA (E_m = −0.175 V at 6.4) and 8ClRfl (E_m = −0.095 V at 6.4). At pH 6.0, 10–20 μM GCD was titrated in the presence of 100 μM MV^{2+} and 1 μM 8ClRfl. For GCD in the presence of acetoacetyl-CoA, the solution concentrations at pH 6.4 were 10–20 μM GCD, 100 μM MV^{2+}, and 1 μM 8ClRfl and 1 μM riboflavin (E_m and −0.149 V).

Potential measurements were made on GCD in the presence of excess CCoA in a manner different than that described above and were done in the presence of the indicator dyes 1 μM 8ClRfl and 3 μM indigo disulfonate (E_m = −0.059 V, pH 6.4). Here, GCD was fully reduced in the presence of the dyes, 1 μM indigo disulfonate, 5 μM PYC, and 1 μM 8ClRfl, and then CCoA was added to a final concentration of 150 μM. Potential values were measured at the equilibrium reached after CCoA addition and after perturbation of this equilibrium by addition of either reductive or oxidative charge.

All potentials are reported versus the standard hydrogen electrode (SHE).

Measurement of the E_m of BCoA/CCoA. The E_m of the BCoA/CCoA couple was measured at pH 6.4 as described by Stankovich and Soltysik (1987). This remeasurement was necessary to assess how the differing buffer composition in these GCD studies might affect the E_m of the couple such that a more accurate comparison of redox properties could be made. In this experiment the initial conditions were that the side arm contained 3 μM BCD while the spectroelectrochemical cell cuvette contained 150 μM each of CCoA and BCoA in pH 6.4 buffer. Also present in the cell were the dyes 20 μM PYC

(E_m = 0.0) and 10 μM indigo trisulfonate (E_m = −0.06 V). The cell contents were reduced until PYC was half-reduced; then, the enzyme was added. Potentials were recorded at the ensuing equilibrium and subsequent returns to equilibrium following perturbation of the system by addition of reductive or oxidative charge.

$\hat{E}$ Measurement. The determinations of $\hat{E}$ for GCD bound to the substrate/product redox couple model BCoA/CCoA were conducted similarly to that described for BCD by Stankovich and Soltysik (1987). A 1:1 ratio of BCoA/CCoA was kept in the side arm of the spectroelectrochemical cell such that when added to the system, the concentration of each was 150 μM. Initially, however, the cuvette contained 9.50 μM GCD, 100 μM MV^{2+}, and 5 μM PYC, only. This solution was degassed, and reductive charge was added until PYC was half-reduced. The enzyme remained fully oxidized through this time. After ΔV was <0.001/min (~0.020 V), the CoA's were added. Perturbations of the resulting equilibrium were performed by alternating additions of reductive and oxidative charge.

Assays. Glutaryl-CoA dehydrogenase was assayed with a phenazine methosulfate mediated dichlorophenolindophenol reduction method as outlined by Husain and Steenkamp (1985). An assay performed before and after a spectroelectrochemical experiment revealed a 16% loss of activity. Thus, unless otherwise stated, experiments were never performed on "used" enzyme.

Dithionite Titrations. Two experiments were performed with sodium dithionite as a chemical reductant of GCD in order to corroborate coulometric experiments involving GCD in the presence of the product, CCoA (see Results). In one experiment, 2.5 mL of 10–20 μM GCD and 150 μM CCoA in pH 6.4 buffer was placed in a cell and degassed. Aliquots of dithionite (standardized against LFA immediately before use) were anaerobically added to the solution to titrate the enzyme–product complex. In the other experiment, 2.5 mL of 10–20 μM GCD in pH 6.4 buffer was half-reduced by addition of dithionite before addition of CCoA from the side arm to a final concentration of 150 μM (this latter experiment was repeated electrochemically with the same chemical concentrations).

Acyl-CoA Titrations. Anaerobic titrations of GCD by GCoA or BCoA were performed with approximately 2.5 mL (GCoA titration) or 4 mL (BCoA titration) of degassed 5–10 μM GCD at pH 6.4. A 1.40 mM solution of GCoA or 2.0 mM BCoA was anaerobically added to the system via a gas-tight syringe.

$K_{d,ox}$ Measurements. Measurements of $K_{d,ox}$ of GCD_{ox} complexation with CCoA or β-hydroxybutyryl-CoA were done aerobically by monitoring the change in the spectra of the oxidized enzyme upon addition of aliquots of 10.3 mM CCoA or 11.78 mM β-hydroxybutyryl-CoA. Difference spectra were used to calculate the individual $K_{d,ox}$ values similarly as in Schopfer et al. (1988).

HPLC Procedure. High-performance liquid chromatography was performed on an Epson EQUITY I+ interfaced Spectra-Physics HPLC system composed of an SP 4290 integrator, an SP 8800 pump, and an SP 8450 UV/vis detector. A Vydac protein and peptide C_{18} column was used to conduct the separation of acyl-CoA compounds according to a modified version of the method of Corkey (1985) in which an isocratic elution of 95% KP_i and 5% methanol was run from 0 to 5 min followed by gradient elution to 30% KP_i and 70% methanol from 5 to 48 min. The solvents, 0.05 M KP_i, pH 5.3, and methanol, were filtered and degassed before use.

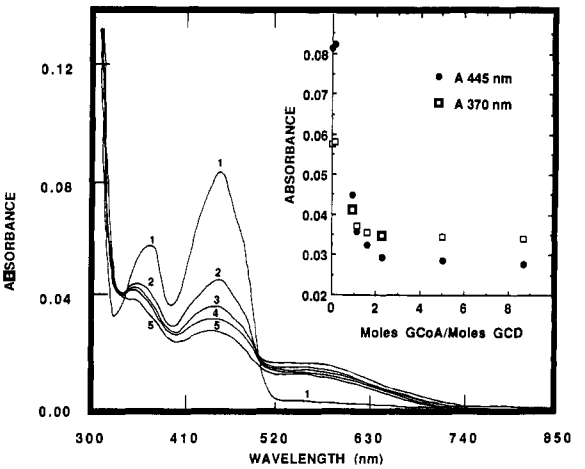

FIGURE 1: Anaerobic GCoA titration of GCD. Enzyme concentration was 6.125 μM (15.3 nmol) in 0.05 M KP_i, pH 6.4, 4 °C, containing 10% ethylene glycol. Stock GCoA concentration was 1.4 mM. Spectra were corrected for dilution and correspond to nanomoles of GCoA added: spectrum 1, 0.0; spectrum 2, 14.0; spectrum 3, 17.5; spectrum 4, 24.6; spectrum 5, 133.0. Intermediate spectra were removed for clarity. Inset shows plot of absorbance vs a ratio of moles of GCoA added to moles of FAD present.

The above HPLC procedure was done to further clarify the composition of the experimental solution in which $\hat{E}$ was measured for GCD in the presence of BCoA and CCoA. Possible eluents and their retention times (min) from this experiment included BCoA (34.6), CCoA (30.0), MV^{2+} (not observed), PYC (38.8), ethylene glycol (not observed), FAD (30.1), and β-hydroxybutyryl-CoA (25.3). Chromatograms were recorded of the simulated experimental $\hat{E}$ sample solution that included all but GCD and also of experimental samples "spiked" with possible eluents such that peak identification was less ambiguous.

Resonance Raman Spectroscopy at 4 °C. Resonance Raman spectra were obtained in the laboratory of Dr. Lawrence Que, Jr., Department of Chemistry, University of Minnesota, with a SPEX 1403 Raman Spectrometer utilizing a Spectra-Physics 2030T argon ion pump laser and a Spectra-Physics 375B dye laser.

More concentrated GCD samples for RR experiments were made by use of Centricon ultrifiltration membrane cones (molecular weight cutoff 50000) purchased from Amicon, Inc, Lexington, MA. In the set of RR experiments performed at 4 °C, stock solutions of 0.5-mL volume were made containing 0.09 mM GCD, 0.1 mM $(NH_4)_2SO_4$, and 0.33 mM acetoacetyl-CoA, in pH 6.4 KP_i buffer, 4 °C, containing 10% ethylene glycol. Immediately before use, GCD samples were centrifuged and Millipore filtered to remove turbidity. Approximately 100 μL of the above stock solution was placed in the RR sample holder (kept at 4 °C), and the following parameters were set to record a spectrum at the excitation wavelength of 571 nm: slit width, 500 mm; power, 280 mW; pump pressure, 90 psi.

Attempts were made to obtain spectra at 488 and 514 nm at 4 °C, but GCD fluorescence contributed too large of a background to the analysis.

Results

Substrate Titrations of GCD. Anaerobic titrations of GCD by its optimal substrate, GCoA, and a substrate analogue, BCoA, are shown in Figures 1 and 2, respectively. In Figure 1, 1 equiv of GCoA is seen to reduce GCD by 59% while addition of excess GCoA results in a 77% reduction of GCD. In addition, a pronounced long-wavelength charge-transfer

Table I: Comparison of the Redox Potential Values (V) of Glutaryl-CoA Dehydrogenase, Butyryl-CoA Dehydrogenase, General Acyl-CoA Dehydrogenase, and Electron-Transfer Flavoprotein at pH 6.4

enzyme	free	radical	inhibitor bound	radical	substrate/product bound	radical
GCD	−0.085	none	−0.129	blue	~+0.03	red
BCD[a]	−0.061	none	−0.161	none	+0.047	none
GACD[b]	−0.076	blue	NA		−0.025	red (B/C)[d]
	−0.100	blue	NA		+0.003	none (Octa/Octe)[e]
	−0.117	blue	NA		NA	
ETF[c]	+0.010					

[a] Fink et al. (1986) and Stankovich and Soltysik (1987). [b] GACD (Lenn, 1989; Gustafson et al., 1986). [c] Husain et al. (1984). [d] B/C = butyryl/crotonyl-CoA's. [e] Octa/Octe = octanoyl-CoA/octenoyl-CoA.

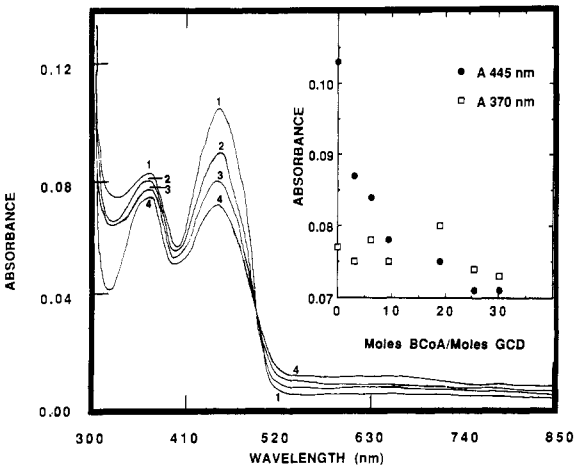

FIGURE 2: Anaerobic BCoA titration of GCD. Enzyme concentration was 7.54 μM (0.029 μmol) in 0.05 M KP_i, pH 6.4, 4 °C, containing 10% ethylene glycol. Stock BCoA concentration was 2.0 mM. Spectra were corrected for dilution and correspond to micromoles of BCoA added: spectrum 1, 0.00; spectrum 2, 0.13; spectrum 3, 0.33; spectrum 4, 1.00. Intermediate spectra were removed for clarity. Inset shows plot of absorbance vs a ratio of moles of BCoA added to moles of FAD present.

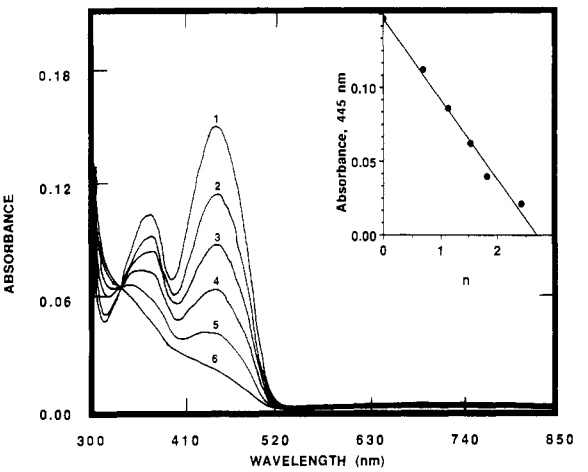

FIGURE 3: Coulometric titration of GCD. Enzyme concentration was 10.8 μM in 0.05 M KP_i, pH 6.4, 4 °C, containing 10% ethylene glycol. Mediator titrant was 100 μM MV^{2+}. Spectrum 1, n = 0.0; spectrum 2, n = 0.7; spectrum 3, n = 1.1; spectrum 4, n = 1.5; spectrum 5; n = 1.8; spectrum 6, n = 2.4. Inset shows plot of absorbance at 445 nm vs n, number of electrons transferred.

band like those seen when GACD and BCD are reduced by their optimal substrates (Thorpe et al., 1979; Stankovich & Soltysik, 1987) is produced. Since Gomes et al. (1981) did not observe decarboxylation of GCoA by GCD from *Pseudomonas fluorescens* in the absence of an electron acceptor for the enzyme, the likely actors (though not confirmed) in this charge-transfer band are glutaconyl-CoA (the "oxidized" GCoA-bound intermediate, $^-OOCCH_2CHCHCOSCoA$) as the acceptor and two-electron-reduced flavin as the charge-transfer donor. There does not appear to be any formation of radical in this titration as the absorbance at 370 nm is seen to decrease with the absorbance at 445 nm (Draper & Ingraham, 1968; Massey et al., 1970). A stoichiometric reduction of 15.3 nmol of GCD would require 15.3 nmol of GCoA, and 133 nmol of GCoA was required to produce the final spectrum in Figure 1, showing that an equilibrium is being reached between GCD_{ox} and GCD_{2e} and reduced and oxidized GCoA.

In the titration of GCD by BCoA, shown in Figure 2, 1 equiv of BCoA reduces GCD by 7%, forming CCoA in the process (Lenich & Goodman, 1986), while the maximum amount of GCD reducible by excess BCoA was 35%. A small amount of what appears to be a reduced GCD–CCoA charge-transfer band at long wavelengths [as described by Husain and Steenkamp (1985), and for GACD by Thorpe et al. (1979)] is formed during the titration. There may be a slight amount of red radical formed in this titration as the absorbance at 370 nm does not decrease significantly with the decrease in the absorbance at 445 nm. This is more easily seen in the inset plot of the absorbance values at 370 and 445 nm versus the ratio of the moles of BCoA added to the moles of FAD present in the enzyme. Absorbance values at 445 nm are seen to decrease at the beginning of the titration and then level off as a large excess of BCoA is added to the GCD solution. In contrast, absorbance values at 370 nm change little as the titration proceeds. A stoichiometric amount of BCoA needed to reduce the 32.4 nmol of GCD in this experiment would be 32.4 nmol, and a total of 1.0 μmol of BCoA was added. Again an equilibrium appears to have been achieved among GCD_{ox}, GCD_{2e}, the substrate (BCoA), and the product (CCoA).

Spectroelectrochemical Titrations of Free GCD. To further clarify the properties of GCD reduction, simple coulometric titrations of free GCD were performed. Figure 3 shows the turbidity-corrected titration of GCD at pH 6.4, and the inset shows an absorbance versus n (the number of electrons used to reduce the enzyme) plot. The calculated current efficiency was 87%. A similar titration was performed for GCD at pH 6.0 (data not shown). Neither titration revealed any indication of radical stabilization by the enzyme as evidenced by the lack of absorbance increase in either the 580-nm region or in the 370-nm region. The molar absorptivities of fully reduced GCD were calculated to be 1960 M^{-1} cm^{-1} at 445 nm and 4200 M^{-1} cm^{-1} at 370 nm from the GCD_{2e} spectrum and from the known values of the molar absorptivities for GCD_{ox}, 13600 M^{-1} cm^{-1} at 445 nm and 9400 M^{-1} cm^{-1} at 370 nm (Husain & Steenkamp, 1985).

Potentiometric titrations of free GCD were conducted in the presence of the indicator dyes 8ClRfl and LFA at pH 6.4 and 8ClRfl at pH 6.0 and were essentially spectrally identical with the titration shown in Figure 3 [data in Byron (1989)]. After correction for dye and turbidity interferences in the data

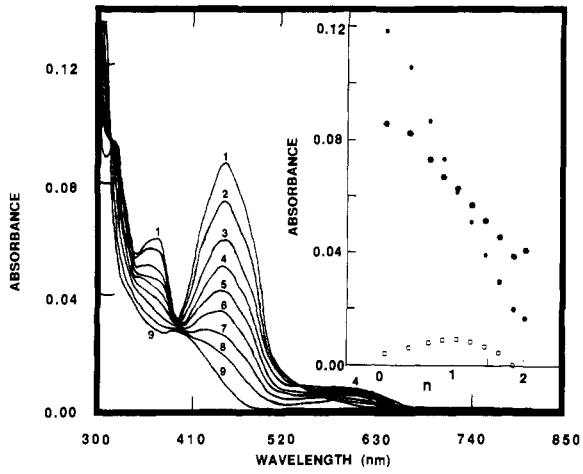

FIGURE 4: Coulometric titration of acetoacetyl-CoA-bound GCD. Enzyme concentration was 9.5 μM in 0.05 M KP_i, pH 6.3, 4 °C, containing 10% ethylene glycol. The mediator titrant was 100 μM MV^{2+}. Inhibitor concentration was 35 μM. Spectrum 1, n = 0.0; spectrum 2, n = 0.4; spectrum 3, n = 0.7; spectrum 4, n = 0.9; spectrum 5, n = 1.1; spectrum 6, n = 1.3; spectrum 7, n = 1.5; spectrum 8, n = 1.7; spectrum 9, n = 2.0. Inset shows absorbance vs n, number of electrons transferred. (●) 445 nm; (*) 370 nm; (□) 590 nm.

analyses, a GCD midpoint potential of −0.085 V was calculated with a Nernst slope of 0.026 V at pH 6.4, and at pH 6.0, a GCD midpoint potential of −0.064 V was calculated with a Nernst slope of 0.026 V (Table I). While the measured values for GCD overlap well with the potential range of the indicator dye 8ClRfl, the spectra of 8ClRfl and LFA are so similar to those of GCD and these dyes are present in such small amounts that it was not possible to independently calculate the enzyme potential from their spectral behavior. However, the slopes calculated from the Nernst plots of the potentiometric data for GCD were nearly ideally Nernstian, thereby granting confidence in the calculated reduction potentials for GCD. The positive shift in the GCD midpoint potential found for the lower pH solution is consistent with the pH dependence of measured reduction potentials for FAD species (Fink et al., 1986; Einarsdottir et al., 1988). However, an extensive study of the pH/potential dependence of free GCD could not be undertaken as the protein tended to precipitate at other pH values.

Spectroelectrochemical Titrations of Acetoacetyl-CoA-Bound GCD. A coulometric titration of GCD in the presence of excess acetoacetyl-CoA is shown in Figure 4. The λ_{max} molar absorptivities of GCD_{ox} and GCD_{2e} have changed to 12 500 and 2450 M^{-1} cm^{-1}, respectively. It is immediately noticeable that the spectral behavior of acetoacetyl-CoA-bound GCD has changed from that in Figure 3 by the absorbance changes occurring in the 570–590-nm region of the titration spectra. This is further illustrated in the inset to Figure 4 in which the absorbance values at 445 and 590 nm are plotted as a function of n. By use of a typical molar absorptivity value for the blue neutral radical form of FAD, 5000 M^{-1} cm^{-1}, the amount of radical stabilized in this coulometric experiment is approximately 20%. Thorpe et al. (1979) calculated a molar absorptivity of 5900 M^{-1} cm^{-1} for the blue radical form of reduced, free GACD, and use of this value here gives a value of 17% stabilized by GCD–acetoacetyl-CoA. These results suggest that the binding of the inhibitor induces a change in the environment at the coenzyme binding site of GCD.

Acetoacetyl-CoA-bound GCD was reduced in the presence of 8ClRfl and riboflavin to measure the E_m of the complex [data in Byron (1989)]. The Nernst analysis revealed an E_m of −0.129 V (Table I), and by use of the same molar absorptivity values as with the coulometric titration, the amount of blue radical stabilized in this potentiometric experiment was 15–17%, slightly less than that calculated for the coulometric analysis of the enzyme. The slope, 0.033 V, is nearly ideally reversible such that the amount of blue radical stabilized by the enzyme is not enough to interfere with the analysis of the two-electron reduction. This negative shift (−0.044 V) in E_m from that measured for free GCD translates into acetoacetyl-CoA binding 29.3 times more tightly to the oxidized form of GCD than to the reduced form, consistent with its observed role as an inhibitor of enzyme activity.

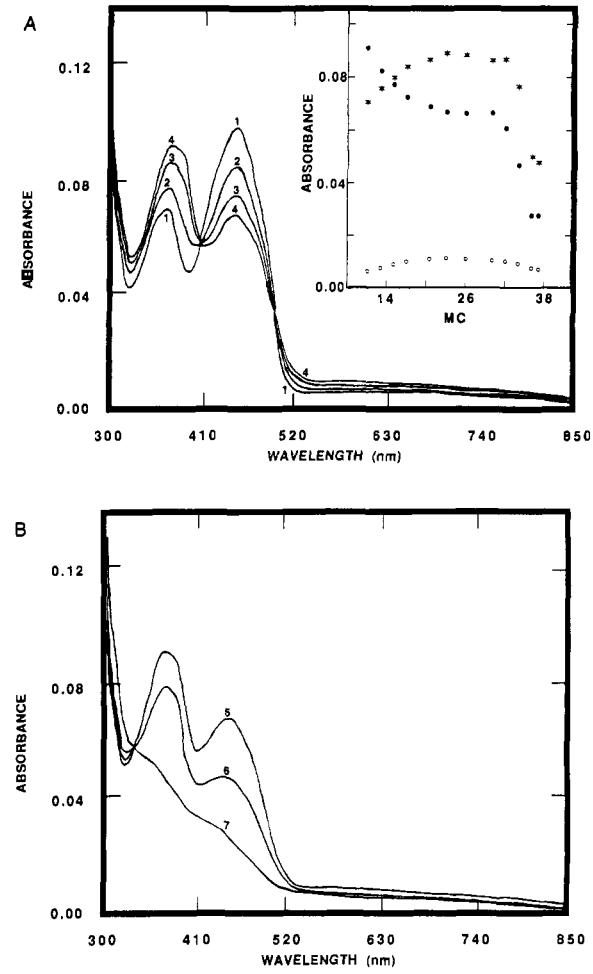

FIGURE 5: Coulometric titration of CCoA-bound GCD. Enzyme concentration was 7 μM in 0.05 M KP_i, pH 6.4, 4 °C, containing 10% ethylene glycol. Mediator titrant was 100 μM MV^{2+}. Product concentration was 150 μM. Spectra 5–7 were taken immediately after addition of charge. Spectra correspond to the number of millicoulombs added to the system: spectrum 1, 0.0; spectrum 2, 13.0; spectrum 3, 17.0; spectrum 4, 23.0; spectrum 5, 26.0; spectrum 6, 32.0; spectrum 7, 36.0. Intermediate spectra were omitted for clarity. Inset shows absorbance vs MC, the number of millicoulombs added to the system. (●) 445 nm; (*) 370 nm; (○) 590 nm.

Reduction of CCoA-Bound GCD. Crotonyl-CoA was observed to complex GCD with a measured K_d of 23 μM (Byron, 1989). Excess CCoA was complexed to GCD, and the system was reduced either coulometrically or chemically with sodium dithionite. These titrations are shown in Figures 5 and 6. It is apparent by the decrease in absorbance in the 445-nm regions, by the concomitant increase in absorbance in the 370-nm regions of the spectra, and by the formation of isosbestic points near 420 and 500 nm that a significant amount of red anionic radical is being stabilized in both titrations as the complex is reduced. It appears to be the predominant species by spectrum 4 in Figure 5a and spectrum 2 in Figure 6.

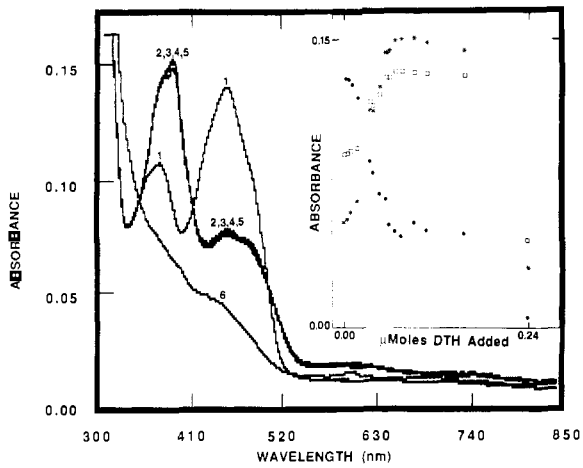

FIGURE 6: Dithionite (DTH) titration of CCoA-bound GCD. Enzyme concentration was 10.22 μM (4 mL) in 0.05 M KP_i, pH 6.4, 4 °C, containing 10% ethylene glycol. Spectrum 1, oxidized CCoA-bound GCD; spectrum 2, 0.065 μmol of DTH added; spectrum 3, 0.073 μmol of DTH added; spectrum 4, 0.106 μmol of DTH added; spectrum 5, 0.155 μmol of DTH added; spectrum 6, 0.237 μmol of DTH added, reduced CCoA-bound GCD. Inset shows a plot of absorbance versus micromoles of DTH added. (●) 445 nm; (*) 380 nm; (□) 370 nm.

The behavior of these titrations was unusual, however, as the enzyme complex resisted reduction to its fully reduced form. At the beginning of the titrations, aliquots of charge or sodium dithionite appeared to reduce GCD normally, but from spectra 4 and 5 in panels A and B of Figure 5 and from spectra 2–5 in Figure 6, aliquots of charge or sodium dithionite appeared to initially produce significant amounts of GCD_{2e}, but the spectra quickly changed back to that corresponding to the red radical form of GCD. The insets to Figures 5A and 6 reflect this behavior as the absorbance values at 445, 370, and 520 nm are plotted against the number of millicoulombs or moles of sodium dithionite added to the system. Normally these plots can be used to estimate the amount of radical stabilized by the enzyme (Stankovich, 1980; Stankovich & Fox, 1983). However, two anomalies can be immediately observed from these plots. One is that the absorbance values in the middle of the plots are not seen to change with an increase in millicoulombs or sodium dithionite added, and the other is that the reduced spectrum is not reached until approximately 38 mC of charge is added to the system in the coulometric titration or 0.237 μmol of sodium dithionite. Theoretically, it should require fewer than 10 mC to reduce 5–10 μM GCD and fewer than 0.05 μmol of sodium dithionite to reduce the same amount. Thus, it would appear that when GCD_{2e} is produced, CCoA reoxidizes the enzyme, forming BCoA in the process. This "reduced form of CCoA", BCoA, is the only possible product here as GCD has not been observed to catalyze the carboxylation of CCoA (Numa et al., 1964). Furthermore, there was little CO_2 dissolved in solution as the system was thoroughly degassed prior to reduction.

To further test this behavior, another experiment was devised in which CCoA was kept in the cell side arm while, in the cell cuvette, GCD was half-reduced by sodium dithionite. At equilibrium, the CCoA was added, and formation of red radical was again observed [data in Byron (1989)].

Addition of excess CCoA to GCD_{2e} in the presence of indigo disulfonate, PYC, and 8ClRfl resulted in immediate, complete reoxidation of GCD_{2e} to GCD_{ox} [data in Byron (1989)]. This system resisted further electrochemical reduction.

Measurement of E_m of the BCoA/CCoA Couple. The reduction potential of the BCoA/CCoA couple was remeasured under the buffer conditions of the experiments performed in this study. At the point of addition of a catalytic amount of BCD to a 1:1 mixture of BCoA and CCoA, the cell potential was measured to be +0.014 V. The subsequent equilibrium potential measured was +0.018 V (stable for >30 min). This equilibrium potential value was again observed after addition of either 4.25 mC of reductive charge or 2 mC of oxidative charge to the system. From these data, the E_m of the BCoA/CCoA couple is considered to be +0.018 V in 0.05 M KP_i buffer, pH 6.4, 4 °C, containing 10% ethylene glycol. This is in good agreement with the value, +0.023 V, calculated from the measured value obtained at pH 7.0, −0.013 V, corrected to pH 6.4 (2 e^-, 2 H^+ transfer; Stankovich & Soltysik, 1987).

$\hat{E}$ Measurement. An experiment to determine the midpoint potential of GCD in the presence of the substrate/product redox model BCoA/CCoA was performed according to the method of Stankovich and Soltysik (1987). This midpoint potential is termed $\hat{E}$ to reflect the fact that the exact binding conditions the enzyme is experiencing are unknown. The results of this experiment are described in detail in Byron (1989). Upon equilibration of the system after addition of the 1:1 mixture of BCoA and CCoA, GCD was reduced 10–20% in the presence of BCoA and CCoA, and a steady-state cell potential of +0.047 V was recorded. Perturbation of this equilibrium by addition of reductive and oxidative charge did not change the resulting measured equilibrium potentials. This experiment was rerun with the same results.

In order to clarify the above results for the determination of the $\hat{E}$ value for GCD, an HPLC analysis was performed in which an aliquot of the solution represented by the equilibrated GCD–CoA system was analyzed. The resulting chromatogram contained four peaks, which were identified through comparison with the retention times of authentic chemicals and by spiking the analyte sample with probable components (Byron, 1989). From those results, peaks corresponding to BCoA, CCoA, and β-hydroxybutyryl-CoA were identified, and it was seen that most of the CCoA had been converted to β-hydroxybutyryl-CoA. These results indicate that GCD exhibits a large amount of hydratase activity under these conditions; however, an approximate $\hat{E}$ value could be calculated according to

$$+0.047 = \hat{E} + (0.059/n)(\log [GCD_{ox}^*/GCD_{2e}^*]) \quad (1)$$

in which n is assumed to be 2 in this experiment and GCD_{ox}^* and GCD_{2e}^* represent the concentrations of GCD in all forms of complexation. The resulting $\hat{E}$ is +0.03 V, a value that should be considered qualitative at best.

Coulometric Titration of β-Hydroxybutyryl-CoA-Bound GCD. A coulometric titration of 14.01 μM GCD in the presence of 550 μM β-hydroxybutyryl-CoA is shown in Figure 7 in which it appears that, like the GCD–CCoA complex, a significant amount of red radical is also being stabilized as the GCD–β-hydroxybutyryl-CoA complex is reduced. A plot (Figure 7, inset) of absorbance changes versus millicoulombs added to the system reveals that this system also reaches a "plateau" of absorbance values with respect to millicoulomb additions, similarly to reductions of CCoA-bound GCD. In addition, it theoretically takes 10.81 mC to reduce the amount of enzyme present in this experiment, but greater than 30 mC of charge was added to this system without fully reducing the complex. The K_d for the complex GCD_{ox}–β-hydroxybutyryl-CoA was measured to be 21.5 μM (Byron, 1989).

Resonance Raman Spectroscopy. To further compare the spectral properties of GCD with those of GACD and BCD, the vibrational spectrum of GCD bound to the inhibitor, acetoacetyl-CoA, was obtained by resonance Raman (RR) spectroscopy. Figure 8 shows the resulting vibrational spec-

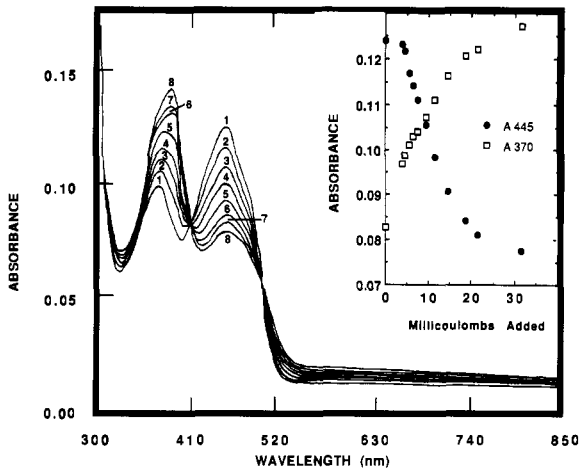

FIGURE 7: Coulometric titration of β-hydroxybutyryl-CoA-bound GCD. Enzyme concentration was 14.01 μM in 0.05 M KP_i, pH 6.4, 4 °C, containing 10% ethylene glycol. Mediator titrant was 100 μM MV^{2+}. Concentration of β-hydroxybutyryl-CoA was 550 μM. Spectra correspond to the number of millicoulombs added to the system: spectrum 1, 4.0; spectrum 2, 6.5; spectrum 3, 9.5; spectrum 4, 11.5; spectrum 5, 14.5; spectrum 6, 18.5; spectrum 7, 21.5; spectrum 8, 31.5. Inset shows a plot of absorbances versus the number of millicoulombs added to the system.

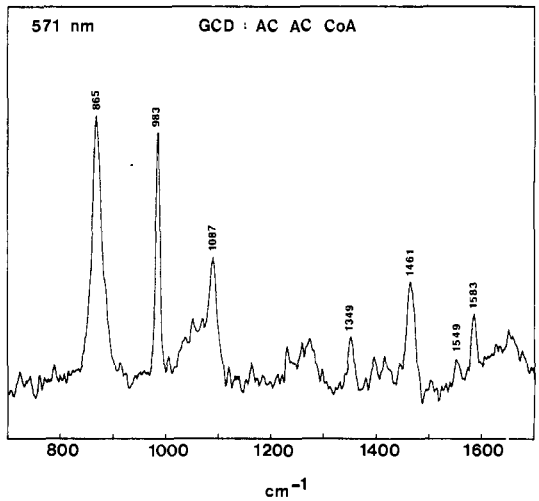

FIGURE 8: Resonance Raman spectrum of acetoacetyl-CoA-bound GCD. Enzyme concentration was 0.90 mM in 0.05 M KP_i, 4 °C, containing 0.1 mM $(NH_4)_2SO_4$. Acetoacetyl-CoA was added to 0.33 mM. Slit width, 500 mm; power, 280 mW; pump pressure, 90 psi. The spectrum is a sum of 13 scans, 5-point digital smoothing applied.

trum of the GCD–acetoacetyl-CoA charge-transfer complex excited at 571 nm. Specific information about the spectrum is located in the figure legend. Bands corresponding to the background are at 865, 983, 1087, and 1461 cm^{-1}. The bands at 1349, 1549, and 1583 cm^{-1} correspond to enhanced flavin RR bands VII, III, and II, respectively, involved in the charge-transfer complex. These bands represent the following vibrational modes as assigned by Abe and Kyoguku (1987) using the standard isoalloxazine ring numbering system: band VII, $\nu(C_{5a}C_6)$ and $\nu(N_{10}C_{10a})$; band III, $\nu(N_1C_{10a})$ and $\nu(C_{4a}N_5)$; band II, $\nu(N_1C_{10a})$ and $\nu(N_{10}C_{10a})$. The same band enhancements were observed when a sample of GCD was excited at 582 nm. How these data compare with those previously reported for GACD (Schmidt et al., 1981) and for BCD (Williamson et al., 1982) will be discussed below.

Discussion

The results of GCD reduction by its substrate, GCoA, and a substrate analogue, BCoA, are similar to those of substrate and substrate analogue reductions of pig kidney GACD (Thorpe et al., 1979). Excess GCoA reduces GCD 77% maximally with no evidence of radical stabilization, and 1 equiv of GCoA reduces GCD by 59%. Maximal reduction of GCD by BCoA is 35% with 1 equiv of GCoA reducing GCD by 7%. With GACD, 1 equiv of its optimal substrate, octanoyl-CoA, reduced the enzyme by 61%, while 1 equiv of BCoA reduced GACD by 31% (Thorpe et al., 1979). The optimal substrate for both GCD and GACD essentially reduces the enzyme by the same amount, but BCoA appears to be a better reductant of GACD than GCD. The reduction of both GCD and GACD by their respective optimal substrates causes the appearance of a prominent long-wavelength charge-transfer band that is not as prevalent when each enzyme is reduced by nonoptimal substrates. Further, BCoA titration of BCD creates significant formation of the long-wavelength charge-transfer band (Stankovich & Soltysik, 1987). Though this significant charge-transfer band is observed in GCoA reduction of GCD, the charge-transfer complex formed with GCD reduction is different from those formed with GACD and BCD reduction. According to Gomes et al. (1981) in a study of the mechanism of the GCD reaction in *Pseudomonas fluorescens*, no decarboxylation of the substrate is observed in the absence of an electron acceptor for reduced GCD. Further, no release of glutaconyl-CoA as a stable solution intermediate has been observed (Numa et al., 1964; Gomes et al., 1981; Husain & Steenkamp, 1985; Lenich & Goodman, 1986). Therefore, it is likely that, in the GCoA reduction of *Paracoccus* GCD, the charge-transfer band observed is not between the enoyl-CoA product and reduced flavin but rather between the oxidized reaction intermediate, glutaconyl-CoA, and reduced GCD-bound flavin. With GACD, it has been shown that the charge-transfer species between GACD and the enoyl-CoA product is the form of the enzyme that reacts with ETF (Hall et al., 1979; Gorelick et al., 1985). If ETF also preferentially reacts with the optimal charge-transfer species of reduced GCD, then ETF must act both to accept electrons and to effect decarboxylation. However, it remains unknown how the reoxidation of GCD proceeds when the species GCD_{2e}–glutaconyl-CoA and GCD_{1e}–CCoA both likely interact with ETF to effect GCD reoxidation. Further, since Ramsay et al. (1987) observed the same kinetic and spectral behavior for ETF interaction with GCD as with GACD, that is, that ETF initially accepts one electron from the GCD–substrate system, it is possible that decarboxylation of glutaconyl-CoA occurs with the abstraction of one electron by ETF. The resulting GCD_{1e}–CCoA complex then reoxidizes similarly to that outlined for GACD by Mizzer and Thorpe (1981) and Gorelick et al. (1985) in which reoxidation of $GACD_{2e}$–enoyl-CoA is said to occur in two, single-electron steps. This decarboxylation scheme is

$$GCD_{ox} + GCoA \rightleftharpoons GCD_{2e}\text{–glutaconyl-CoA} \quad (2)$$

$$GCD_{2e}\text{–glutaconyl-CoA} + ETF \rightleftharpoons GCD_{1e}\text{–CCoA} + CO_2 + ETF^{\cdot-} \quad (3)$$

$$GCD_{1e}\text{–CCoA} + ETF \rightleftharpoons GCD_{ox}\text{–CCoA} + ETF^{\cdot-} \quad (4)$$

Kinetic studies of CO_2 evolution in tandem with spectral monitoring of ETF may further this discussion.

That little or no radical was seen in either the GCoA or the BCoA titration of GCD is similar to results obtained for the coulometric and potentiometric titrations of free GCD. In both cases no radical stabilization of GCD was observed in contrast to the generalization by Massey and Hemmerich (1980) that flavoprotein dehydrogenases stabilize the blue neutral radical form of FAD when reduced. In addition, this lack of radical

stabilization by reduced GCD is in marked contrast to the results of reductive titrations of free GACD, in which blue radical was thermodynamically stabilized during the experiment (Thorpe et al., 1979). The GCD results are also in contrast with those of free BCD, which kinetically stabilizes a small amount of blue radical (Fink et al., 1986). Unlike GACD and BCD, as free GCD is reduced, it appears to be unable to accept a hydrogen bond from a protonated N-5 position of FAD, thus destabilizing the blue radical form of FAD. It would also appear that the degree of blue radical stabilization by a reduced ACD may depend on the relative hydrophobicity of the ACD active site as the active site of GCD must accommodate the highly polar $-COO^-$ group of GCoA while the active sites of BCD and GACD are optimal for relatively hydrophobic short and medium alkyl chains. Such an argument could be strengthened by data on the redox properties of the long-chain ACD, but as yet, no data are available.

Another manifestation of the active site differences among GCD, GACD, and BCD is spectrally observed when acetoacetyl-CoA-bound GCD is reduced. Here, approximately 20% of the blue neutral form of FAD is stabilized by GCD. No observable formation of radical is seen when either acetoacetyl-CoA-bound GACD at pH 8.5 or acetoacetyl-CoA-bound BCD at pH 7.0 is reduced (Mizzer & Thorpe, 1981; Fink, et al., 1986). It is possible the acetoacetyl-CoA binding has allowed an amino acid side chain in GCD to be moved into a position in which it can accept a hydrogen bond from the protonated N-5 of the FAD blue radical, while the ability of GACD and BCD to stabilize the blue neutral radical appears to have been blocked by acetoacetyl-CoA.

Clearly, spectral evidence has been shown here that active site differences exist among GCD, GACD, and BCD. Moreover, these differences are enhanced as the complex is reduced, indicating that ACD substrate specificity is indeed manifested in the redox properties of each enzyme as suggested by Thorpe (1990). It may be that, in general, the enzyme structural changes that occur as GCD is reduced differ from those of GACD and BCD in that the GCD active site must accommodate the highly polar carboxyl end group of GCoA. Once decarboxylation has occurred in the GCD reaction sequence, however, the enzyme likely becomes more "GACD-like", taking on the structural features of the β-oxidation enzymes in order to effect electron transfer to ETF or $ETF^{\bullet-}$ and release of its product, CCoA, into the β-oxidation pathway.

The above argument is strengthened by the results of coulometric and dithionite reductions of CCoA-bound GCD at pH 6.4. Consistent with that observed by Mizzer and Thorpe (1981) for dithionite reduction of CCoA-bound GACD at pH 7.6–8.6, reduction of GCD in the presence of a large excess of CCoA reveals that the red anionic radical form of FAD is stabilized by the enzyme. At the midway points in these titrations, this red radical is the predominant species of GCD in solution. Red radical formation is also seen when CCoA is added to half-reduced free GCD (Byron, 1989). Therefore, such evidence may indicate that the reoxidation of GCD by ETF occurs in successive one-electron transfers similarly to that reported for GACD reoxidation (Mizzer & Thorpe, 1981; Gorelick & Thorpe, 1984; Gorelick et al., 1985). Further, Ramsay et al. (1987) have shown that pig liver ETF stabilized red radical when reduced by the *Paracoccus* GCD/GCoA system just as it does when ETF is reduced by the pig liver or pig kidney GACD/octanoyl-CoA system. They have also shown that the kinetics of the ETF reaction is unchanged whether the *Paracoccus* GCD/GCoA system is used or the pig liver GACD/octanoyl-CoA system is used. These studies validate the suggestion made earlier that reoxidation of GCD/product occurs in a "GACD-like" manner.

The large excesses of reducing equivalents needed to fully reduce GCD in the presence of CCoA are partially consistent with that seen when Mizzer and Thorpe (1981) used an excess of dithionite to reduce GACD in the presence of CCoA. They attributed this finding to the observation that CCoA is capable to reoxidizing $GACD_{2e}$ but not $GACD_{1e}$, and thus as dithionite is added, GACD becomes "locked" in the red radical form, apparently unable to effect intrasubunit electron transfer. It is likely that a similar mechanism is occurring with GCD for at least the first half of the coulometric titration in Figure 5 (spectra 1–4). However, in the second half of the titrations shown in Figures 5 and 6, the reduction of the radical to GCD_{2e} was spectrally observed and was then followed by an immediate reoxidation to the radical form. This apparent one-electron-reoxidation process was puzzling in that CCoA is assumed to reoxidize GCD_{2e} via a two-electron process, producing GCD_{ox} and BCoA [Numa et al. (1964) saw no evidence of GCD catalyzing the recarboxylation of CCoA]. A plausible reason for this is that GCD_{ox}–BCoA quickly exchanges to GCD_{ox}–CCoA under excess CCoA conditions and then a comproportionation reaction between GCD_{ox}–CCoA and GCD_{2e}–CCoA occurs to form $2GCD_{1e}$–CCoA, a thermodynamically stable complex. This comproportionation scheme is

$$GCD_{1e}\text{–CCoA} + MV^{\bullet+} \text{ (or DTH)} \rightleftharpoons GCD_{2e}\text{–CCoA} \quad (5)$$

$$GCD_{2e}\text{–CCoA} \rightleftharpoons GCD_{ox}\text{–BCoA} \quad (6)$$

$$CCoA + GCD_{ox}\text{–BCoA} \underset{\text{fast}}{\rightleftharpoons} GCD_{ox}\text{–CCoA} + BCoA \quad (7)$$

$$GCD_{ox}\text{–CCoA} + GCD_{2e}\text{–CCoA} \rightleftharpoons 2GCD_{1e}\text{–CCoA} \quad (8)$$

in which evidence for step 4 is strongly supported by the coulometric and dithionite titrations shown in Figures 5 and 6 and by an experiment described under Results where red radical formation is observed upon addition of CCoA to an approximately 50:50 mixture of GCD_{ox} and GCD_{2e}. This latter type of experiment was not performed by Mizzer and Thorpe (1981) with GACD, and thus, no comparison of GACD with GCD is available. Evidence for this comproportionation reaction catalyzed by GCD bound to CCoA in the second half of a reductive titration and possibly also in the first half of the titration suggests that intraflavin electron transfer may be more favorable with GCD than with GACD. That such a CCoA-mediated comproportionation reaction occurs with GCD is surprising, but it does not appear to be contradicted by data previously presented for GACD.

This comproportionation mechanism could also explain why little or no red radical is seen when GCD is reduced by either BCoA or a 1:1 mixture of BCoA and CCoA. As a probable equal competitor ($K_d \sim 31$ μM for $GACD_{ox}$–BCoA; Schopfer et al., 1988) with CCoA for GCD_{ox}, BCoA may prevent the comproportionation mechanism from contributing to the spectrally observed equilibrium. In addition, since this comproportionation reaction is observed for the second half of the titration, these latter two experiments may not apply as in each case GCD is reduced less than halfway.

Somewhat contradictory is the experiment in which no GCD radical is observed when a 10-fold excess of CCoA is added to fully reduced GCD. It is likely that the excess CCoA present completely oxidizes GCD_{2e} to GCD_{ox} with the system spending little or no time as a mixture of oxidation states; thus, the comproportionation reaction is not able to contribute to the equilibrium. Therefore, the proposed CCoA-mediated

comproportionation mechanism is consistent with all the available data. This mechanism could be tested with stopped-flow studies of GACD, BCD, and GCD, thus determining whether these reactions of GCD differ markedly from those of the other ACDs.

A summary of the spectroelectrochemical behavior of GCD is contained in Table I. Also included for comparison are some data found for BCD from *M. elsdenii*, pig kidney GACD, and pig liver ETF in which the reduction potential values reported have been corrected to values expected at pH 6.4, the pH of GCD in most of this study. This was done with the 0.030 V/pH unit dependence found for BCD (Fink et al., 1986) for both BCD and GACD and by assuming a 0.030 V/pH unit dependence for the ETF midpoint potential.

From Table I the E_m value, −0.085 V, for free GCD is between those of BCD and GACD, which is consistent with their similar roles of acyl-CoA reduction. The midpoint potential of GCD is shifted negatively from −0.085 to −0.129 V when GCD is complexed to the inhibitor, acetoacetyl-CoA, indicating that acetoacetyl-CoA binds 29.4 times more tightly to oxidized GCD than to reduced GCD, consistent with its role as an inhibitor of GCD activity. This negative shift of midpoint potential is also consistent with the negative shift of the midpoint potential of BCD when complexed to acetoacetyl-CoA though the shift is not as large (Fink et al., 1986).

Measurement of the shift in midpoint potential of GCD in the presence of GCoA and glutaconyl-CoA would have been an ideal vehicle for comparing such data with the midpoint potential shift observed for BCoA- and CCoA-bound BCD from *M. elsdenii* by Stankovich and Soltysik (1987). Their work showed that while the E_m of the CCoA/BCoA couple is −0.018 V, the E_m of free BCD is −0.079 V at pH 7.0, thus creating the appearance of a thermodynamically unfavorable reaction between reduced substrate and oxidized enzyme. When the E_m of BCD is remeasured in the presence of a 1/1 mixture of BCoA and CCoA, the E_m is seen to shift positively to the value −0.015 V. This $\hat{E}$ value indicates not only that the reaction is now thermodynamically favorable but that it is also energy efficient. Such an approach was not possible for GCD with GCoA and glutaconyl-CoA because addition of an indicator dye to the solution would provide the system with an electron acceptor and thus an irreversible decarboxylation reaction would have occurred, thereby interfering with the analysis. Therefore, a model substrate/product couple, BCoA and CCoA, was chosen for this analysis as previous evidence showed that GCD was expected to catalyze a redox equilibrium between the two from the titration of GCD by BCoA, from the reoxidation of GCD_{2e} by CCoA, and from the work of Gomes et al. (1981) in which GCD from *Pseudomonas fluorescens* was seen to catalyze the exchange (FAD N-5 mediated) of the β-hydrogen of GCoA in the absence of an electron acceptor.

As with BCD and GACD, the reduction potential value measured for GCD, −0.085 V, is too negative for electron transfer to occur from BCoA (E_m = +0.018 V) to the enzyme, yet the enzyme was reduced by BCoA in the straightforward titration described for Figure 2. This could only happen if the reduction potential of GCD is shifted positively in the presence of substrate. While the apparently large hydratase activity of GCD (Numa et al., 1964) mars the analysis of the potential of GCD in the presence of the substrate/product model, BCoA/CCoA, reduced GCD was readily observed at a potential well positive of the −0.085-V midpoint potential value measured for free GCD. Therefore, the concept of the thermodynamic regulation of β-oxidation of fatty acids found with BCD and GACD through the "turning on or off" of the enzyme by the presence or absence of substrate is likely extended to an enzyme (GCD) that lies in the pathway of amino acid degradation.

Table II: Comparison of the Resonance Raman Bands for the Charge-Transfer Complexes of Acetoacetyl-CoA-Bound Glutaryl-CoA Dehydrogenase, Butyryl-CoA Dehydrogenase, and General Acyl-CoA Dehydrogenase

enzyme	excitation, λ (nm)	I[e]	II	III	VI	VII
GCD	571		1583	1549		1349
GCD	582		1583	1547		1353
BCD[a]	632.8	1626	1584	1553		1352
GACD[b]	582		1586	1550	1409	1353
RBP[c]	488	1631	1584	1548	1407	1355
FAD[d]	488	1629	1582	1548	1408	1353

[a] Williamson et al. (1982). [b] Schmidt et al. (1981). [c] Bowman and Spiro (1981). [d] Schmidt et al. (1981). [e] Band numbering system is from Bowman and Spiro (1981); units are cm^{-1}.

When GCD_{ox} was titrated with the hydratase product, β-hydroxybutyryl-CoA, alone, the $K_{d,ox}$ was found to match closely that of CCoA binding of GCD_{ox}, such that neither is likely preferred by GCD_{ox}. However, it is unexpected that reduction of GCD in the presence of a large excess of β-hydroxybutyryl-CoA would result in stabilization of significant amounts of red radical. Interestingly, the measured cell potential, +0.025 V, of the reduced GCD (radical) complex suggests that β-hydroxybutyryl-CoA binding may be shifting the midpoint potential of GCD positively. Further consideration of these phenomena will require additional electrochemical experimentation.

Obviously, exploring methods of minimizing the hydratase activity of GCD may improve the analysis, but efforts to do so with GACD and BCD have not completely eliminated this activity (Ellison & Engel, 1986; Lau et al., 1986). It is obviously a large factor in the study of GCD, and it may be worth a future rigorous study among several ACDs as to the kinetic nature of this reaction and as to which oxidation state of the ACD is optimum for hydratase activity.

Studies of the redox properties and the rapid reaction kinetics of ACDs in the presence of substrate and product analogues that do not undergo oxidation and reduction may also aid in the clarification of the above data. Powell et al. (1987) have shown that acyl-CoA thioethers are very good analogues for the study of ACD mechanisms, and in the study of the GCD mechanism, CoA thioethers with a carbonyl or carboxyl group at the end of the carbon chain may serve as better mechanistic probes.

An effort was undertaken to probe the active site differences among GCD, GACD, and BCD through use of resonance Raman (RR) spectroscopy on the charge-transfer species formed between the acceptor, ACD-bound FAD, and the donor, the enolate resonance structure of acetoactyl-CoA. Table II summarizes the RR bands seen for GCD, BCD, and GACD complexes with acetoacetyl-CoA. Data are also given for FAD- and riboflavin-binding protein for comparison. Bands enhanced for each ACD complex are similar for each enzyme, indicating that the vibrational modes of oxidized FAD involved with acetoacetyl-CoA in each charge-transfer complex are invariant. These observations may further corroborate evidence given by Powell et al. (1987) that the binding energies of acyl-CoA thioethers of varying chain lengths to oxidized GACD do not significantly differ. Future use of RR spectroscopy to probe the reduced forms of these enzymes may produce some more indicative results about the environment of bound FAD.

It appears that the spectral and redox properties of GCD are similar enough to pig kidney GACD and BCD from *M. elsdenii* to further allow studies performed on the latter two enzymes to guide the study of GCD. Obviously, however, there exists some additional nonredox function of GCD that allows for the decarboxylation of GCoA that is difficult to probe electrochemically. Alternative means of study of the GCD reaction may shed more light on this step in the sequence. As more amino acid sequences are learned and more X-ray crystallographic studies are performed on ACDs, hopefully GCD will be counted among them, and questions raised by this study can be addressed.

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