The Function of Arg-94 in the Oxidation and Decarboxylation of Glutaryl-CoA by Human Glutaryl-CoA Dehydrogenase*

Received for publication, August 22, 2000, and in revised form, October 5, 2000
Published, JBC Papers in Press, October 6, 2000, DOI 10.1074/jbc.M007672200

Timothy M. Dwyer‡, K. Sudhindra Rao‡, Jonna B. Westover§, Jung-Ja P. Kim¶, and
Frank E. Frerman**

From the Department of Pediatrics, Human Medical Genetics Program, and The Department of Pharmaceutical Sciences, The University of Colorado Health Sciences Center, Denver, Colorado 80262 and Department of Biochemistry, The Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Glutaryl-CoA dehydrogenase catalyzes the oxidation and decarboxylation of glutaryl-CoA to crotonyl-CoA and CO₂. Inherited defects in the protein cause glutaric acidemia type I, a fatal neurologic disease. Glutaryl-CoA dehydrogenase is the only member of the acyl-CoA dehydrogenase family with a cationic residue, Arg-94, situated in the binding site of the acyl moiety of the substrate. Crystalllographic investigations suggest that Arg-94 is within hydrogen bonding distance of the γ-carboxylate of glutaryl-CoA. Substitution of Arg-94 by glycine, a disease-causing mutation, and by glutamine, which is sterically more closely related to arginine, reduced kₐₑ₅₄ of the mutant dehydrogenases to 2-3% of kₒₑ₅₄ of the wild type enzyme. Kₐₚ of these mutant dehydrogenases for glutaryl-CoA increases 10- to 16-fold. The steady-state kinetic constants of alternative substrates, hexanoyl-CoA and glutaramyl-CoA, which are not decarboxylated are modestly affected by the mutations. The latter changes are probably due to steric and polar effects. The dissociation constants of the non-oxidizable substrate analogs, 3-thiaglutaryl-CoA and acetoacetyl-CoA, are not altered by the mutations. However, abstraction of α-proton from 3-thiaglutaryl-CoA, to yield a charge transfer complex with the oxidized flavin, is severely limited. In contrast, abstraction of the α-proton of acetoacetyl-CoA by Arg-94 → Gln mutant dehydrogenase is unaffected, and the resulting enolate forms a charge transfer complex with the oxidized flavin. These experiments indicate that Arg-94 does not make a major contribution to glutaryl-CoA binding. However, the electric field of Arg-94 may stabilize the dienions resulting from abstraction of the α-proton of glutaryl-CoA and 3-thiaglutaryl-CoA, both of which contain γ-carboxylates. It is also possible that Arg-94 may orient glutaryl-CoA and 3-thiaglutaryl-CoA for abstraction of an α-proton.

Human glutaryl-CoA dehydrogenase (GCD)† is a homotetrameric mitochondrial flavoprotein that catalyzes the α,β-dehydrogenation and decarboxylation of glutaryl-CoA, an intermediate in the oxidation of lysine and tryptophan (1–3). The products of the reaction are crotonyl-CoA and CO₂ (2). The introduction of the double bond requires abstraction of an α-proton, presumably the pro-Rα-hydrogen, by analogy with other acyl-CoA dehydrogenases (4, 5), followed by hydride transfer from the β-carbon to the flavin and decarboxylation of the enzyme-bound intermediate glutaconyl-CoA (5). Investigations of related bacterial glutaryl-CoA dehydrogenases indicate that decarboxylation requires electron transfer from the dehydrogenase to its electron acceptor, electron transfer flavoprotein, or an artificial electron acceptor capable of accepting one electron (5, 6). Recent studies of the human enzyme indicate that Glu-370 is the catalytic base that abstracts the α-proton (7). Previous work by Gomes et al. (5) with GCD from Pseudomonas fluorescens suggests that the α-proton is subsequently transferred from the conjugate acid of the catalytic base to the crotonyl-CoA anion following decarboxylation of the γ-carboxylate. The decarboxylation reaction catalyzed by GCD is unique among the acyl-CoA dehydrogenases.

Glutaric acidemia type I is an inherited neurologic disease resulting from defects in GCD (1). An Arg-94 → Gly mutation has been identified as a disease-causing mutation in one patient with glutaric acidemia type I, suggesting that Arg-94 plays an important role in the reaction catalyzed by the dehydrogenase (8). Arg-94 is located at the “bottom” of the substrate-binding site (9). This position is occupied by a glycine residue in many acyl-CoA dehydrogenases (10) or, in the case of medium chain acyl-CoA dehydrogenase, by a glutamine residue (9–11). With the exception of Arg-94 in human glutaryl-CoA dehydrogenase, no acyl-CoA dehydrogenases contain a cationic residue in the active site (10). Based on the x-ray structure of the dehydrogenase, it was proposed that Arg-94 participates in the binding of glutaryl-CoA. It was also suggested that the delocalized charge of the guanidinium group might also stabilize a transient crotonyl-CoA anion prior to protonation or might stabilize, developing a negative charge at the γ-carbon in the transition state for decarboxylation (9).

In this report, we have investigated the functions of Arg-94 and Ser-98 (Arg-138 and Ser-142 in the complete sequence containing the 44-amino acid mitochondrial targeting sequence (3)). We determined steady-state kinetic constants of wild type GCD, Arg-94 → Gly, Arg-94 → Gln, and Ser-98 → Ala mutants of GCD using glutaryl-CoA and two alternate substrates, hexanoyl-CoA and glutaramyl-CoA, that are not decarboxylated. We also investigated the binding of non-oxidizable glutaryl-CoA analogs, 3-thiaglutaryl-CoA and acetoacetyl-CoA, by wild type GCD and the mutants. These studies suggest that Arg-94

* This work was supported by grants from the United States Public Health Service (NS39339 to F. E. F. and GM29076 to J. J. P. K.) and by a grant from the Denver Children’s Hospital Research Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** To whom correspondence should be addressed: Dept. of Pediatrics, Box C235, University of Colorado Health Sciences Center, 4200 East Ninth Ave., Denver, CO 80262 Tel.: 303-315-7269; Fax: 303-315-8080; E-mail: frank.frerman@uchsc.edu.

† The abbreviations used are: GCD, glutaryl-CoA dehydrogenase; PGHS, prostaglandin H synthase; FeFPP₅, ferrocenium hexafluorophosphate.

This paper is available on line at http://www.jbc.org
functions to effectively decrease the pKₐ of the α-proton in the low dielectric in the interior of the protein. Electrostatic stabilization by Arg-94 would favor formation of α-anions of the substrate and substrate analog, 3-thiaglutaryl-CoA, both of which contain γ-carboxylates. Arg-94 may also orient the substrate for efficient proton abstraction at the α-carbon from glutaryl-CoA. Arg-94 apparently does not play a major role in the binding of the substrate. Ser-98 also lies at the bottom of the active site and is conserved in several members of the acyl-CoA dehydrogenase family (long chain, α-methyl-butyryl-, and short chain acyl-CoA dehydrogenases) (10). Glu-99 occupies a similar position in the three-dimensional structure of medium-chain acyl-CoA dehydrogenase and participates in a hydrogen bonding network with tightly bound water molecules at the base of the active site in the absence of acyl-CoA ligands (11). Substitution of Ser-98 by alanine has little effect on the steady-state kinetic constants of the dehydrogenase. The latter data suggest that the polarity of the steady-state kinetic constants of the dehydrogenase. The latter

### EXPERIMENTAL PROCEDURES

#### Materials—
Ferrocenium hexafluorophosphate (FcPF₆) was obtained from Aldrich. CoASH, glutaryl-CoA, acetocetoy-CoA, and hexanoyl-CoA were from Sigma. [1,5-14C]Glutaric acid was from ICN Biochemicals and [1,5,14C]glutaryl-CoA was prepared from the anhydride as described (2). Glutaric acid (glutaric acid monoamide) was synthesized as described by Marquez et al. (12) from glutaric anhydride and NH₃. Synthetic glutaramyl-CoA had a melting point of 93 °C in agreement with the literature value, 93–94 °C (13), and the 1H NMR spectrum was as expected (12). Glutaramyl-CoA was synthesized by the amidinylation method (14) and purified by high pressure liquid chromatography (7). 3-Thiaglutaryl-CoA was synthesized as described by Dwyer et al. (7). Porcine medium chain acyl-CoA dehydrogenase was purified as described previously (15).

#### Purification of Proteins—
Wild type GCD and the Arg-94 → Gly and Ser-98 → Ala mutants were expressed in *Escherichia coli* and purified as described (7). The absorption spectrum of Arg-94 → Gly GCD (λ₂₇₀ nm: A₃₄₅ nm: 5:3.0:65 ± 1.00) was very similar to that of the wild type dehydrogenase (λ₂₇₀ nm: A₃₄₅ nm: 5:0.0:62 ± 1.00). The Arg-94 → Gln mutant of GCD was expressed in *E. coli* grown at 23 °C. Optimal expression was dependent on co-expression of the chaperones, GroEL/ES, from the plasmid reported by Gatesby (16). Arg-94 → Gln was purified as described previously (7), except that the gradient on DEAE-Sepharose was extended from 150 to 170 mM potassium phosphate, pH 7.0. The ratios of absorption maxima of the purified Arg-94 → Gln GCD were A₂₇₀ nm/A₃₄₅ nm: 5:3.0:67.1:0.0. The extinction coefficients of FAD at the visible absorption maxima, 446 and 447 nm, in wild type, Arg-94 → Gly, and Arg-94 → Gln dehydrogenases are 14.5 × 10³ (7), 14.2 × 10³, and 14.9 × 10³ M⁻¹ cm⁻¹, respectively. The latter values were determined after release of the FAD from the proteins with 0.1% sodium lauryl sulfate (17). The errors in these determination were ±0.6%. The Ser-98 → Ala mutant dehydrogenase was expressed and purified as described previously.

#### Enzyme Assays—
The GCD proteins were assayed at 25 °C in 10 mM potassium phosphate, pH 7.6, with 200 µM FcPF₆ as the electron acceptor. Glutaryl-CoA, hexanoyl-CoA, and glutaramyl-CoA were the varied substrates. Enzyme activity was routinely determined using ε_{390 nm} = 4.3 × 10⁵ M⁻¹ cm⁻¹ for FcPF₆ (18). GCD specific activity was also assayed by following the release of 14CO₂ from [1,5-14C]glutaryl-CoA (2) in the standard reaction containing 10 mM potassium phosphate, pH 7.6, 25 µM [1,5-14C]glutaryl-CoA, and 100 µM FcPF₆.

#### Site-directed Mutagenesis—
Site-directed mutagenesis was carried out using the QuikChange mutagenesis system (Stratagene) according to the manufacturer’s instructions. The mutagenized plasmids were sequenced in the region of the mutations to verify introduction of the mutation. Cassettes containing the mutated site were removed by digestion with EcoRI and SacI and ligated into the wild type plasmid that had been digested with the same endonuclease. The expression vectors were then sequenced across junctions and through the mutared cassette to demonstrate the mutation and the absence of unwanted mutation. No unwanted mutations were detected. DNA was sequenced using the dideoxynucleotide termination method (19) using the Alf express system (Amersham Pharmacia Biotech).

### RESULTS

#### Steady-state Kinetic Constants of Wild Type and Mutant GCDs with Glutaryl-CoA and Alternate Substrates—
In the initial experiments, we determined the steady-state kinetic constants of wild type GCD and the three mutant dehydrogenases, Arg-94 → Gly, Arg-94 → Gln, and Ser-98 → Ala with glutaryl-CoA, hexanoyl-CoA, and glutaramyl-CoA (Table I). Decarboxylation is obviously not a step in the steady-state turnover of hexanoyl-CoA and glutaramyl-CoA. All reactions were assayed in 10 mM potassium phosphate, pH 7.6, with FcPF₆ as the electron acceptor.

#### k₅₆ of the Arg-94 → Gly mutant with glutaryl-CoA as substrate decreases to about 3% that of the wild type dehydrogenase, and Kₘ for glutaryl-CoA increases 16-fold. Glutamine was also substituted for Arg-94, because glutamine is sterically similar to arginine, and the amide group maintains the polar character at the mutated site, although the cationic charge is absent. Modeling of glutaryl-CoA into the active site suggests that Arg-94 is within hydrogen bonding distance of the γ-carboxylate of glutaryl-CoA. However, the γ-carboxylate side chain is at least 2.3 Å shorter than that of arginine; therefore, the γ-carboxylate of glutaryl-CoA and the amide of Gln-94 cannot interact as strongly as in the wild type dehydrogenase. Substitution of Arg-94 by glutamine increases Kₘ for glutaryl-CoA 10-fold, and k₅₆ decreases to 2% of k₅₆ of wild type GCD. However, the kinetic constants of Ser-98 → Ala GCD with

|                | Wild type GCD | Arg-94 → Gly GCD | Arg-94 → Gln GCD | Ser-98 → Ala GCD |
|----------------|---------------|------------------|------------------|-----------------|
| Kₘ (μM)        | 2.6 ± 0.4     | 4.11 ± 0.4       | 25.4 ± 2.4       | 1.4 ± 0.2       |
| k₅₆ (s⁻¹)      | 9.0 ± 0.4     | 0.24 ± 0.01      | 0.22 ± 0.01      | 10.3 ± 0.3      |
| Kₘ (hexanoyl-CoA) (μM) | 3.5            | 0.006            | 0.009            | 7.3             |
| k₅₆ (hexanoyl-CoA) (s⁻¹) | 36.8 ± 6.0     | 20.1 ± 0.4       | 5.1 ± 0.1        | 40.3 ± 5.4      |
| k₅₆ (glutaryl-CoA) (s⁻¹) | 18.5 ± 1.2     | 14.2 ± 0.6       | 8.5 ± 0.4        | 25.3 ± 1.7      |
| k₅₆ (glutaramyl-CoA) (μM) | 0.5            | 21.2 ± 3.0       | 7.9 ± 1.8        | 3.4 ± 1.1       |
| k₅₆ (glutaramyl-CoA) (s⁻¹) | 1.6 ± 0.3      | 6.4 ± 0.2        | 1.5 ± 0.2        | 2.7 ± 0.3       |
| k₅₆ (glutaryl-CoA) (s⁻¹) | 2.2 ± 0.2      | 1.4             | 0.3              | 0.2             |
| k₅₆ (glutaramyl-CoA) (s⁻¹) | 1.4            | 0.6             | 0.8              |                |
glutaryl-CoA as substrate are very similar to those of wild type GCD (Table I). Ser-98 apparently plays no major role in the steady-state kinetic pathway of the dehydrogenase.

In contrast with these results, the steady-state kinetic constants of the enzymes are less affected with hexanoyl-CoA as the substrate. The 45% decrease in $K_m$ of the Arg-94 $\rightarrow$ Gly mutant may be attributed to the increased volume at the bottom of the active site, which is likely occupied by water molecules in the mutant protein. The mutation would eliminate an unfavorable interaction between the charged guanidinium group of Arg-94 and the alkyl chain of hexanoyl-CoA.

The increased volume of the active site of the mutant may be attributed to the increased volume at the bottom of the active site. The specificity of the enzyme reflected by $k_{cat}/K_m$ is essentially unchanged when compared with the wild type GCD. By the same criterion, the specificity of the Arg-94 $\rightarrow$ Gln mutant actually increases. Moreover, the kinetic constants of this mutant enzyme are very similar to those of medium-chain acyl-CoA dehydrogenase in which a glutamine residue occupies the same position as Arg-94 in GCD (11, 20).

The residues comprising the active sites of GCD and medium-chain acyl-CoA dehydrogenases are reasonably conserved with the exception of Arg-94 and a negatively charged residue, Glu-99, in medium-chain acyl-CoA dehydrogenase (10, 11, 21). Glutaryl-CoA is not a substrate for medium-chain acyl-CoA dehydrogenase, perhaps due to the unfavorable interaction of Glu-99 with the γ-carboxylate of glutaryl-CoA. The $K_m$ of Arg-94 $\rightarrow$ Gln GCD for hexanoyl-CoA decreases further with the elimination of Arg-94, and the cavity in Arg-94 $\rightarrow$ Gly GCD, presumably containing bound water, is occupied by the side chain of glutamine. $k_{cat}$ of this mutant with hexanoyl-CoA does decrease relative to that of wild type GCD, perhaps due to the decreased dissociation of product as in medium chain acyl-CoA dehydrogenase (22) or positioning of substrate.

The $K_m$ for wild type GCD for glutaramyl-CoA is similar to the $K_m$ for glutaryl-CoA, but $k_{cat}$ decreases. The structural similarity of the resulting 2,3-enoyl-CoA amide analog product with the tightly bound natural intermediate, glutaroyl-CoA, may limit steady-state turnover due to a slow off-rate. The increased volume of the active site and decreased strength of hydrogen bonding of the γ-amide may cause the increase of $K_m$ for glutaryl-CoA, but the increased volume of the active site of Arg-94 $\rightarrow$ Gly GCD may promote the rate of dissociation of the 2,3-enoyl-CoA product. The $K_m$ for glutaramyl-CoA of Arg-94 $\rightarrow$ Gln is increased relative to wild type GCD but is significantly less than the Arg-94 $\rightarrow$ Gly mutant, which has the large cavity at the bottom of the active site. The substitution of glutamine at position 94 yields a mutant with a turnover that is similar to the wild type dehydrogenase, perhaps limiting turnover by decreasing the rate of dissociation of the glutaroyl-CoA analog. The specificity of the Arg-94 $\rightarrow$ Gln mutant is essentially identical to that of the Arg-94 $\rightarrow$ Gly mutant with the γ-amide analog substrate likely reflecting the absence of interaction between the amide and Arg-94 seen in the wild type dehydrogenase.

The predominant effect of both substitutions for Arg-94 in GCD with glutaryl-CoA as substrate is to reduce $k_{cat}$ to 2–3% of the wild type turnover, although $K_m$ for glutaryl-CoA increases about 10- to 16-fold. The effects of the mutations of Arg-94 on the steady-state kinetic constants could be ascribed to a role in the binding of the γ-carboxylate, or, perhaps, to stabilization of the transient crotonyl-CoA carbanion prior to protonation to yield crotonyl-CoA (9). Stabilization of such a negatively charged intermediate is not required in the oxidation of either hexanoyl-CoA or glutaramyl-CoA. The effects of the mutations can also be evaluated from the resulting changes in $k_{cat}/K_m$ (Table I). Glutaryl-CoA is, predictably, the best substrate of wild type GCD. When glycine or glutamine is substituted for Arg-94, the values of $k_{cat}/K_m$ indicate that hexanoyl-CoA is the best of the three substrates and that the mutant enzymes are only slightly less efficient with glutaramyl-CoA as substrate. $k_{cat}/K_m$ of the mutants with glutaryl-CoA as substrate is strongly influenced by the large decrease of $k_{cat}$, again suggesting the possible involvement of the delocalized positive charge of the guanidinium group in stabilizing the transient crotonyl-CoA anion.

The decrease in $k_{cat}$ does not seem to be of sufficient magnitude to indicate a role for Arg-94 in the stabilization of a developing negative charge at the γ-carbon in a decarboxylation transition state. It is important that the Arg-94 $\rightarrow$ Gly and Arg-94 $\rightarrow$ Gln mutants are capable of catalyzing decarboxylation in the standard radiochemical assay (Table II). Although not extrapolated to infinite substrate concentration, the decreased specific activities are of the same magnitude as the decreases in $k_{cat}$ shown in Table I. Thus, Arg-94 is not absolutely required for decarboxylation of glutaroyl-CoA.

These experiments also suggest a catalytic basis for the pathogenicity of the Arg-94 $\rightarrow$ Gly mutation in humans (8). Substitution of lysine for Arg-94 is also a disease-causing mutation (23); however, this mutant subunit was unstable when expressed in E. coli, and the mutation may affect the folding pathway of the mutant subunit. The stability of this subunit in human cells is not known.

**Table II**

| Enzyme         | Activity (% wild type) |
|----------------|------------------------|
| Wild type      | 3.1 ± 0.1              |
| Arg-94 $\rightarrow$ Gly | 0.030 ± 0.003 (0.9) |
| Arg-94 $\rightarrow$ Gln  | 0.010 ± 0.004 (0.3) |

**Binding and Deprotonation of 3-Thiaglutaryl-CoA and Acetoacetyl-CoA by Wild Type, Arg-94 $\rightarrow$ Gly, and Arg-94 $\rightarrow$ Gln GCDs**—The function of Arg-94 was further investigated by determining the effects of the Arg-94 $\rightarrow$ Gly and Arg-94 $\rightarrow$ Gln mutations on the binding of 3-thiaglutaryl-CoA, a nonoxidizable analog of glutaryl-CoA (7). The obvious difference between the wild type and mutants is that binding of the analog to the mutant proteins yields no detectable charge transfer complex with a maximum at about 825 nm that is observed in the titration of the wild type protein (Fig. 1, A–C). The charge transfer species presumably results from the interaction of the α-carbanion of 3-thiaglutaryl-CoA following abstraction of a α-proton of the analog with the electron deficient oxidized flavin (24, 25). In the case of the Arg-94 $\rightarrow$ Gln dehydrogenase a weak charge transfer band ($\lambda_{max}$ $\sim$ 650 nm) may be present (Fig. 1C). The analog binds to both mutant proteins as demonstrated by the perturbation of the flavin spectra of the two mutants proteins, accompanied by a 7-nm blue shift in the spectra. The concentration-dependent binding of 3-thiaglutaryl-CoA to the wild type, Arg-94 $\rightarrow$ Gly, and Arg-94 $\rightarrow$ Gln dehydrogenases is shown in Fig. 1 (A–C). The $K_d$ values of complexes of 3-thiaglutaryl-CoA with wild type GCD and the two mutants are given in Table III. These data indicate that Arg-94 is not a major determinant for glutaryl-CoA binding, because its absence has no effect on the dissociation constants of the complexes with mutant proteins when compared with the wild type. Rather, both mutations alter the equilibrium for the abstraction of the α-proton of 3-thiaglutaryl-CoA.

Proton abstraction from 3-thiaglutaryl-CoA by a Gly-370 $\rightarrow$ Asp mutant of GCD is also significantly decreased (≤97%) (7). Glu-370 is the catalytic base in GCD that abstracts the α-pro-
Spectrophotometric titration were carried out in 125 mM potassium phosphate, pH 7.6, 25°C.

| Enzyme                  | Dissociation constant | Stoichiometry, ligand/flavin |
|-------------------------|-----------------------|-----------------------------|
| Wild type               | 6.7 ± 1.2             | 0.91 ± 0.04                 |
| Arg-94 → Gly            | 8.5 ± 2.1             | 0.99 ± 0.06                 |
| Arg-94 → Gln            | 10.4 ± 4.3            | 1.03 ± 0.10                 |

FIG. 1. Spectrophotometric titrations of wild type, Arg-94 → Gly, and Arg-94 → Gln GCDs with 3-thiaglutaryl-CoA. The spectra are numbered and indicated in parentheses with the concentrations of ligand. A, the wild type GCD, 9.1 μM, was titrated with 3-thiaglutaryl-CoA (in μM) as follows: (1) 0; (2) 2.0; (3) 5.9; (4) 9.9; (5) 16.8; (6) 32.7; (7) 44.5; (8) 56.4. B, Arg-94 → Gly GCD, 12.2 μM, was titrated with 3-thiaglutaryl-CoA (in μM) as follows: (1) 0; (2) 2; (3) 6; (4) 14; (5) 30; (6) 60. C, Arg-94 → Gln GCD, 14.4 μM, was titrated with 3-thiaglutaryl-CoA (in μM) as follows: (1) 0; (2) 2; (3) 6; (4) 18; (5) 72; (6) 192. All titrations were conducted at 25 °C in 125 mM potassium phosphate, pH 7.6. Some spectra are omitted for clarity but all spectral data were used to calculate the data shown in Table III.

drogenases. Arg-94 → Gly and Arg-94 → Gln mutations affect proton abstraction from 3-thiaglutaryl-CoA but do not significantly alter the $K_a$ values. Arg-94 may facilitate abstraction of the $\alpha$-proton by neutralization of the negative charge of the $\gamma$-carboxylate, effectively decreasing the $pK_a$ of the $\alpha$-proton in the low dielectric of the active site. Arg-94 may also function to orient the substrate for efficient abstraction of the $\alpha$-proton.

Wild type GCD binds acetoacetyl-CoA with a submicromolar $K_a$ and abstracts the $\alpha$-proton of the 3-ketoacyl-CoA with formation of the enzyme-bound enolate (7). The wild type dehydrogenase-enolate complex exhibited decreased absorbance at 447 nm due to perturbation of the flavin upon acetoacetyl-CoA binding, increased absorbance at 322 nm due to the enolate, and increased absorbance in the 550-nm region due to formation of the charge transfer complex between the enolate and electron-deficient oxidized flavin (7). Titration of the Arg-94 → Gln GCD with acetoacetyl-CoA (Fig. 2) yields results that are very similar to those obtained with wild type GCD (7). Acetoacetyl-CoA, lacking the $\gamma$-carboxylate, would not be expected to require Arg-94 to facilitate deprotonation of an $\alpha$-proton, for binding, or orientation of the ligand with respect to Glu-370. The $K_a$ of the complex is 0.1 μM, and the stoichiometry of binding is 0.87 per mol of enzyme flavin. The values for wild type GCD are 0.4 and 0.90 μM in 50 mM potassium phosphate at the same pH (7).

DISCUSSION

The three-dimensional structure of human GCD suggested that Arg-94 could play a role in the binding of glutaryl-CoA and glutaconyl-CoA in the oxidative decarboxylation of glutaryl-CoA catalyzed by the enzyme (2). When glutaryl-CoA was modeled into the active site of the enzyme, the $\gamma$-carboxylate is within hydrogen bonding distance of the guanidinium group of Arg-94 (9). The possibilities were also considered that Arg-94 could stabilize a transition state involving developing a negative charge at the $\gamma$-carbon prior to decarboxylation or stabilize the intermediate crotonyl-CoA anion (9). The experiments presented here show that the major effect of substitution of Arg-94 by glycine or glutamine is on $k_{cat}$ of the mutant enzymes. Both mutant enzymes have about 2–3% residual activity with glutaryl-CoA as substrate, but have smaller effects on $K_m$ for glutaryl-CoA. It is reasonable to conclude that Arg-94 does not participate in stabilization of a decarboxylation transition state, because the decrease in $k_{cat}$ is at least an order of magnitude less than might be expected for such an effect. When decarboxylation does not enter steady-state turnover, as with hexanoyl-CoA and glutaramyl-CoA, substitution at position 94 has comparatively little effect on the steady-state kinetic constants of the enzyme. It is of interest that the dehydrogenating activity of GCD determined with hexanoyl-CoA is 2-fold greater than the turnover of glutaryl-CoA, although the chemical steps for $\alpha,\beta$-dehydrogenation are essentially identical. We speculate that the greater intrinsic dehydrogenation activity with the alternate substrate indicates that decarboxylation is rate-limiting in the turnover of glutaryl-CoA. The decarboxylation of glutaconyl-CoA and complete turnover involves oxidation of the
dehydrogenase flavin, decarboxylation, protonation of the crot-
onyl-CoA anion intermediate, and product dissociation.

A likely function of Arg-94 is to effectively decrease the pKₐ of the α-proton of glutaryl-CoA and 3-thiaglutaryl-CoA. The α-anions of glutaryl-CoA and 3-thiaglutaryl-CoA are in close proximity to the γ-carboxylates. Electrostatic stabilization would facilitate the formation of the α-anion in the low dielec-
tric of the enzyme interior. This hypothesis is supported by kinetic data that show that substitution of Arg-94 has compar-
atively little effect on the steady-state kinetic constants of the mutant enzymes with alternative substrates that do not con-
tain a γ-carboxylate group. In the same vein, the charge trans-
fer complex between the 3-thiaglutaryl-CoA anion with the dehydrogenase flavin is not stabilized by the Arg-94 → Gly
Arg-94 → Gln mutants. In contrast, the Arg-94 → Gln mutant deprotonates acetoacetyl-CoA and stabilizes the enolate. Ab-
ence of the γ-carboxylate permits deprotonation of acetoacetyl-
CoA in the absence of Arg-94.

A second possible function of Arg-94 in GCD may be similar to that of the conserved arginine residue, Arg-120, in prosta-
glandin endoperoxides 1 (PGHS-1) and the homologous Arg-
106 in PGHS-2. The steady-state kinetic constants and struc-
tures of the two proteins are almost identical. The crystal
structures of ovine PGHS-1 and human and murine PGHS-2 have been determined with substrate analogs (non-steroidal anti-inflammatory drugs) bound in the active sites (26–29). The carboxylate of the ligands interacts with the conserved arginine. Site-directed mutagenesis of Arg-120 alters the steady-state kinetic constants of the mutants, and additional kinetic analyses of human Arg-106 → Gln PGHS-2 with alternate fatty acid substrates suggested that Arg-106 might position the substrate for abstraction of the 13-pro-S-hydrogen (30, 31). Furthermore, the ratio of products of the Arg-120Gln and Arg-120Leu mutants of human PGHS-2 is altered so that Arg-106 appears to position arachidonate such that bis-oxygen-
ation is favored over monooxygenation (31). Similarly, Arg-402 has been proposed to function in the binding and positioning of arachidonate in human 15-lipoxygenase (32).

In the case of the Gln and Gly substitutions of Arg-94 in GCD, the Kₐ values increase only 10- to 16-fold, respectively, and decreases in kₐcat/Kₐm values of the mutant enzymes are dominated by the 50-fold decreases of kₐcat. The decreased kₐcat could be due to the positioning of the substrate with respect to Glu-370, the catalytic base, or with respect to the flavin so that the efficiency of hydride transfer also decreases. Substitution of aspartate for Glu-370 in glutaryl-CoA dehydrogenase results in a similar decrease of kₐcat as that determined for the Arg-94 → Gly and Arg-94 → Gln mutants (7). Dwyer et al. (7) proposed that the decreased kₐcat of the Glu-370 → Asp is due to the unfavorable equilibrium of the abstraction of the α-proton based on similar studies with 3-thiaglutaryl-CoA. The binding of the non-oxidizable substrate analogs, 3-thiaglutaryl-CoA and acetoacetyl-CoA, support the idea that Arg-94 positions the substrate for efficient deprotonation. The Arg-94 → Gly and Arg-94 → Gln mutant proteins bind the non-oxidizable sub-
strate analog, 3-thiaglutaryl-CoA, with affinity equal to the wild type, but abstraction of the α-proton is severely limited. A role in positioning the substrate for hydride transfer is more difficult to determine, because the initial step in the reaction pathway of the Glu-370 → Asp mutant is compromised. Also consistent with conclusions from kinetic data with alternative substrates, the binding and deprotonation of acetoacetyl-CoA by Arg-94 → Gln GCD is essentially unaffected by the mutation. The evidence for this is based on the appearance of the charge transfer complex when the enolate is formed on the enzyme. Finally, preliminary data indicates that oxidized hu-
man glutaryl-CoA dehydrogenase has enoyl-CoA hydratase ac-
tivity and hydrates glutaconyl-CoA, the tightly bound interme-
tate in the reductive half-reaction of the dehydrogenase flavin.2 Paracoccus denitrificans GCD and pig kidney medium-
chain acyl-CoA dehydrogenase also exhibit enoyl-CoA hy-
dratase activity with crotonyl-CoA (6, 33). Hydratase activity of Arg-94 → Gln GCD with glutaconyl-CoA as substrate is re-
duced 30-fold. These data suggest that Arg-94 also positions glutaconyl-CoA for hydration in the active site of the dehydrogenase.

To summarize, Arg-94 does not appear to play a major role in the binding of glutaryl-CoA and, probably, glutaconyl-CoA. Other residues in the active site and the presumed hydrogen bonds of the thioester oxygen with the 2'-hydroxyl of the FAD and peptide amide hydrogen of Glu-370 (9) apparently play more significant roles. The data can be interpreted to indicate that Arg-94 electrostatically facilitates deprotonation of substrate and 3-thiaglutaryl-CoA. Arg-94 may also function to orient the substrate to facilitate proton abstraction. It is not clear whether Arg-94 is required for positioning glutaryl-CoA for hydride transfer to the flavin or stabilization of the transient crotonyl-CoA anion.

REFERENCES

1. Goodman, S. I., and Frerman, F. E. (1995) in The Metabolic and Molecular Basis of Inherited Disease (Scriver, C. R., Baudet, A. L., Sly, W., and Valle, D., eds) Vol. I, pp. 1451–1460, McGraw-Hill, New York
2. Lenich, A. C., and Goodman, S. I. (1986) J. Biol. Chem. 261, 4090–4096
3. Goodman, S. I., Kratz, L. E., DiGiulio, K., Biery, B. J., Goodman, K. E., Isaya, G., and Frerman, F. E. (1995) Hum. Mol. Genet. 4, 1493–1498
4. Tharpe, C., and Kim, J.-J. P. (1995) FASEB J. 9, 718–725
5. Gomes, B., Fendrich, G., and Abeles, R. H. (1981) Biochemistry 20, 1481–1490
6. Byron, C. M., Stankovich, M. T., and Husain, M. (1990) Biochemistry 29, 3691–3700
7. Dwyer, T. M., Rao, K. S., Goodman, S. I., and Frerman, F. E. (2000) Biochem-
istry 39, 11488–11499
8. Goodman, S. I., Stein, D. E., Schlesinger, S., Christensen, E., Schwartz, M., Cole, R. D., Gleenberg, C. R., and Elpeleg, O. N. (1990) Hum. Mutat. 12, 141–144
9. Kim, J.-J. P., Wang, M., Paschke, R., Goodman, S. I., Biery, B. J., and Frerman, F. E. (1999) in Flavins and Flavoproteins 1999 (Ghisa, S., Krouneck, P., Macheroxus, P., and Sund, H., eds) pp. 539–542, Rudolf Weber, Berlin, Germany
10. Telford, E. A., Moynihan, L. M., Markham, A. F., and Lenich, N. J. (1999) Biochim. Biophys. Acta 1416, 371–376
11. Kim, J.-J. P., Wang, M., and Paschke, R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7523–7527
12. Marquez, V. E., Kelley, J. A., and Driscoll, J. S. (1980) J. Org. Chem. 45, 5308–5312
13. Jeffery, G. H., and Vogel, J. (1934) J. Chem. Soc. 1101–1105

2 J. B. Westover and F. E. Frerman, unpublished data.
Arg-94 in Human Glutaryl-CoA Dehydrogenase

14. Bernert, J. T., Jr., and Sprecher, H. (1977) J. Biol. Chem. 252, 6736–6744
15. Frerman, F. E., Kim, J.-J. P., Huhta, K., and McKeen, M. C. (1980) J. Biol. Chem. 255, 2195–2198
16. Gatenby, A. A. (1992) Plant Mol. Biol. 19, 677–687
17. McKeen, M. C., Beckmann, J. D., and Frerman, F. E. (1983) J. Biol. Chem. 258, 1866–1870
18. Lehman, T. C., and Thorpe, C. (1990) Biochemistry 29, 10594–10602
19. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463–5467
20. Ghisla, S., Braunworth, A., and Vock, P. (1997) in Flavins and Flavoproteins 1996 (Stevenson, K. J., Massey, V., and Williams, C. H., Jr., eds) pp. 629–632, University of Calgary Press, Calgary, Canada
21. Vock, P., En gst, S., Eder, M., and Ghisla, S. (1998) Biochemistry 37, 1848–1860
22. Kumar, N. R., Peterson, K. L., and Srivastava, D. K. (1997) in Flavins and Flavoproteins 1996 (Stevenson, K. J., Massey, V., and Williams, C. H., Jr., eds) pp. 633–636, University of Calgary Press, Calgary, Canada
23. Zschocke, J., Quak, E., Guldberg, P., and Hoffmann, G. F. (2000) J. Med. Genet. 37, 177–181
24. Lau, S.-M., Brantley, R. K., and Thorpe, C. (1988) Biochemistry 27, 5089–5095
25. Tamaoki, H., Nishina, Y., Shiga, K., and Miura, R. (1999) J. Biochem. (Tokyo) 125, 285–296
26. Garavito, R. M., and DeWitt, D. L. (1999) Biochim. Biophys. Acta 1441, 278–287
27. Picot, D., Loll, P. J., and Garavito, R. M. (1994) Nature 367, 243–249
28. Loll, P. J., Picott, D., Ekabo, O., and Garavito, R. M. (1996) Biochemistry 35, 7330–7340
29. Kurumai, R. G., Stevens, A. M., Gierse, J. K., McDonald, J. J., Stegeman, R. A., Pak, J. Y., Gildehaus, D., Miyasiro, J. M., Penning, T. D., Seibert, K., Isakson, P. C., and Stallings, W. C. (1996) Nature 384, 644–648
30. Bhattacharyya, D. K., Lecomte, M., Rieke, C. J., Garavito, M., and Smith, W. L. (1996) J. Biol. Chem. 271, 2175–2184
31. Rieke, C. J., Mulichak, A. M., Garavito, R. M., and Smith, W. L. (1999) J. Biol. Chem. 274, 17109–17114
32. Gan, Q.-F., Browner, M. F., Sloane, D. L., and Sigal, E. (1996) J. Biol. Chem. 271, 25412–25416
33. Lau, S.-M., Powell, P., Buettner, H., Ghisla, S., and Thorpe, C. (1986) Biochemistry 25, 4184–4189