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Conversion of a decarboxylating to a non-decarboxylating glutaryl-coenzyme A dehydrogenase by site-directed mutagenesis

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ABSTRACT

Glutaryl-coenzyme A (CoA) dehydrogenases (GDHs) are acyl-CoA dehydrogenases, which usually dehydrogenate and decarboxylate the substrate to crotonyl-CoA. In some anaerobic bacteria, non-decarboxylating GDHs exist that release glutaconyl-CoA (2,3-dehydroglutaryl-CoA) without decarboxylation. The differing mechanisms of decarboxylating and non-decarboxylating GDHs were investigated by site-directed mutagenesis of the gene coding for the crotonyl-CoA-forming GDH from *Geobacter metallireducens*. Exchange of single amino acids involved in substrate carboxylate binding impaired the decarboxylation step, resulting in relative glutaconyl-CoA:crotonyl-CoA formation rates of 1:1 (S97A) or 13:1 (Y370A). The total amount of glutaconyl-CoA formed was maximal in the Y370V+S97A double mutant. The results obtained indicate that an invariant deprotonated Tyr plays a crucial role for optimizing the leaving group potential of CO_2 in decarboxylating GDHs.

1. Introduction

Glutaryl-coenzyme A (CoA) dehydrogenases (GDHs) are members of the FAD-containing family of acyl-CoA dehydrogenases, which usually dehydrogenate the substrate to an α,β-unsaturated enoyl-CoA with an oxidized electron transferring flavoprotein as electron acceptor. GDHs are unique in decarboxylating the dehydrogenated intermediate glutaconyl-CoA (2,3-dehydroglutaryl-CoA) to crotonyl-CoA [1,2] (Fig. 1). The mechanism of GDHs has been elucidated in a number of studies with the human enzyme GDH_{hum} [3–8]. The dehydrogenation step is initiated by the abstraction of the *pro-R* α-proton from the substrate by a catalytic glutamate base, followed by a hydride transfer from the β-carbon to the flavin cofactor. The subsequent decarboxylation step of the unsaturated intermediate glutaconyl-CoA involves the cleavage of the C4–C5 bond (Fig. 1), yielding a crotonyl-CoA dienolate anion intermediate and CO_2. After protonation of the former by the conserved glutamic acid residue, the product is released.

In anaerobic bacteria that degrade aromatic compounds, two different types of GDHs exist [9–12]. Denitrifying or Fe(III)-respiring bacteria, for example *Geobacter metallireducens*, employ a decarboxylating, crotonyl-CoA forming GDH. In contrast, sulfate reducing bacteria, for example *Desulfococcus multivorans,* employ a non-decarboxylating, glutaconyl-CoA forming enzyme (GDH_{Des}; Fig. 1B). In the latter organism, decarboxylation of glutaconyl-CoA is catalyzed by membrane-bound, sodium ion-pumping decarboxylases that contain a biotin cofactor [13]. The low energy yield is suggested to necessitate sulfate-reducing bacteria to conserve the energy of the exergonic decarboxylation step ($\Delta G^{\circ\prime} = -30$ kJ mol^{-1}).

Very recently, the crystal structure of the non-decarboxylating GDH from *D. multivorans* (GDH_{Des}) was solved in the presence of glutaconyl-CoA [14]. Structure alignments with GDH_{hum} in complex with the glutaconyl-CoA analogue 4-nitrobut-2-enoyl-CoA [5] revealed marked structural differences in the vicinity of the carboxylate/nitro groups (Fig. 1): (i) In decarboxylating GDH_{hum}, an invariant arginine residue (Arg94) forms a monodentate, in GDH_{Des} (Arg87) a bidentate complex with the substrate carboxylate. (ii) A glutamate residue (E87 in GDH_{hum}), present only in decarboxylating GDHs, was assumed to weaken the Arg-guanidinium/substrate-carboxylate interaction. (iii) Only decarboxylating GDHs contain conserved tyrosine and serine residues (Tyr369 and Ser95 in GDH_{hum}) in the active site that are replaced by Val366 and Val88, respectively, in GDH_{Des}. Both are in hydrogen bond distances to each other and to the substrate carboxylate. The presence of Tyr369 and Ser95 was suggested to disable the formation of a

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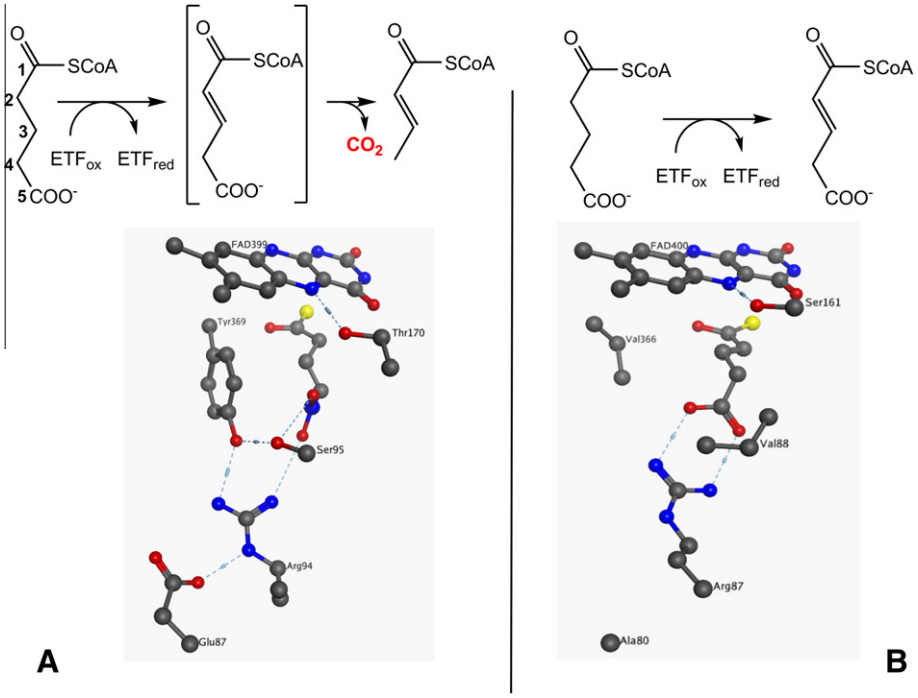

Fig. 1. Reactions catalyzed and amino acid residues involved in substrate carboxylate binding in decarboxylating GDH$_{hum}$ and non-decarboxylating GDH$_{Des}$. (A) Reaction and active site of GDH$_{hum}$ with bound 4-nitrobut-2-enoyl-CoA; the glutaconyl-CoA intermediate (in brackets) is not released during the reaction. In the decarboxylating GDH$_{Geo}$ the numbering of the corresponding amino acids is: Glu89, Ser97, and Tyr370. ETF = electron transferring flavoprotein. (B) Reaction and active site of GDH$_{Des}$ with bound glutaconyl-CoA.

tight bidentate guanidinium/carboxylate complex and to position the carboxylate group for protonation of the postulated dienolate anion intermediate by a glutamic acid residue [14].

Attempts to provide experimental evidence for these predictions by site-directed mutagenesis of GDH$_{Des}$ failed. The corresponding V366Y, Val88S, or A80E mutants greatly lost the FAD cofactor [14]. While some of them retained a very low residual dehydrogenation activity, the formation of crotonyl-CoA was never observed. To gain deeper mechanistic insights into the different functions of decarboxylating and non-decarboxylating GDHs, the opposite approach was performed in this work: using GDH from *G. metallireducens* (GDH$_{Geo}$), the conversion of a decarboxylating into a non-decarboxylating GDH was attempted by site-directed mutagenesis.

2. Materials and methods

2.1. Multiple sequence alignment

In a multiple sequence alignment, the sequences of five decarboxylating and two non-decarboxylating GDHs were compared employing the ClustalW program [15]. The comparison of the active sites of decarboxylating and non-decarboxylating GDHs was carried out by superimposition of monomers of the enzymes of *Homo sapiens* (PDB:1SIQ/1SIR), *Thermus thermophilus* (PDB: 2EBA), and *D. multivorans* (PDB:3MPI) using the software MOE (2008.10, Chemical Computing Group, Inc., Montreal, QC).

2.2. Site-directed mutagenesis

Mutations were introduced into the gene of GDH$_{Geo}$ (gi78194537) by PCR reactions using the Quik-Change site-directed mutagenesis kit (revision B, Stratagene), as described previously [12]. The primers used for the individual mutations are listed in Table S1. For single mutations a wild-type gene containing plasmid served as template. For insertion of further point mutations, the mutated plasmids served as template. The correctness of the generated variant genes coding for GDH$_{Geo}$ was verified by sequencing. Wild-type and mutated genes contained an additional sequence coding for six His at the C-terminus.

2.3. Heterologous expression, purification, and FAD content of wild type and mutated GDH$_{Gmet}$

Wild type and variant genes coding for GDH$_{Gmet}$ were heterologously expressed in *Escherichia coli*. Afterwards, the gene products were purified using a Ni-Sepharose high-performance affinity column as described in Ref. [12]. Expression and purification was monitored by SDS–PAGE. The amount of bound FAD cofactor was determined by absorption spectroscopy at 450 nm using the molar extinction coefficient given in Ref. [12]. Since the majority of GDH variants had greatly lost the cofactor, reconstitution of the cofactor-free enzyme was carried out by adding 0.5 mM FAD and incubation for 30 min at 30 °C. Unbound FAD was removed using a PD-10 desalting column (GE Healthcare).

2.4. Enzyme assays and determination of kinetic parameters

GDH activities were routinely determined in a continuous spectrophotometric assay following the time-dependent reduction of ferrocenium hexafluorophosphate as electron acceptor [12]. The K_m- and V_{max}-values for the glutaryl-CoA dehydrogenation activities were determined by this assay. For the determination of the substrate/products of GDH variants, a discontinuous assay was carried out, in which the consumption of glutaryl-CoA and the formation of glutaconyl-CoA and/or crotonyl-CoA were followed by HPLC

as described in Ref. [12]. The initial rate of glutaconyl-CoA formation in non-decarboxylating GDH_{Geo} variants was estimated by this assay.

3. Results

The Glu87, Ser95, and Tyr369 residues in GDH_{hum} are conserved in decarboxylating GDHs but are replaced by Ala and Val residues in the non-decarboxylating GDH_{Des} (Fig. 1), [14]. We studied the anticipated role of these three amino acids as distinguishing elements between decarboxylating and non-decarboxylating GDHs in GDH_{Geo}. The corresponding residues Glu89, Ser97, and Tyr370 were converted into Ala, Val and Phe residues by site-directed mutagenesis. To identify possible cumulative effects of individual amino acid exchanges, double mutations were constructed. In all cases, soluble proteins were obtained, which migrated on SDS-gels according to the expected mass of approximately 44.5 kDa including the His-tag. The FAD-content, K_m- and V_{max}-values for glutaryl-CoA and glutaconyl-CoA-forming activities are summarized in Table 1; representative results from HPLC assays are presented in Fig. 2.

In agreement with earlier results, wild type GDH_{Geo} catalyzed the dehydrogenation and decarboxylation of glutaryl-CoA to crotonyl-CoA and CO_2, not even traces of the glutaconyl-CoA intermediate were identified [12]. Using almost saturating concentrations of the substrate, the reaction followed pseudo-first-order kinetics (Fig. 2A). In the molecular variants discussed below, the dehydrogenation activity was negatively affected in all molecular variants. Moreover, the reaction did not follow Michaelis–Menten-kinetics probably due to the disturbance of the coupled multistep process. For this reason, the K_m- and V_{max}-values determined have to be interpreted as apparent.

The interaction of E89 with R96 (E87 with R94 in GDH_{hum}, Fig. 1) was expected to weaken the guanidinium–carboxylate binding. Consequently, the E89A exchange was predicted to strengthen the electrostatic interaction between the invariant Arg-guanidinium and the substrate-carboxylate, which in turn was assumed to decrease the decarboxylation activity. However, glutaryl-CoA was dehydrogenated and decarboxylated to crotonyl-CoA with virtually no release of glutaconyl-CoA (Table 1). While the FAD-cofactor content and K_m were hardly affected, the dehydrogenation activity was 50-fold lower than in the wild type.

The hydroxyl group of Ser97 in GDH_{Geo} should be in a hydrogen bond distance to the substrate carboxylate. For this reason, its conversion to Val or Ala was expected to decrease the decarboxylation partial activity. Both mutant enzymes were colorless, and the FAD content could be reconstituted to maximally 26%. Whereas a glutaryl-CoA-converting activity was hardly detectable in the S97V mutant enzyme, the S97A variant showed about 5% of the wild type enzyme activity, accounting for approximately 20% when corrected for the FAD content. The K_m-value was even lower than in the wild type enzyme (Table 1). Most importantly, the formation of both crotonyl-CoA and glutaconyl-CoA was observed with the mutant enzyme as evidenced by HPLC-analysis (Fig. 2B). The initial rate of glutaconyl-CoA and crotonyl-CoA formation was equal within the first 5 min. The latter increased after prolonged incubation, while the former decreased. In addition, the dehydrogenation rate determined in the spectrophotometric assay also increased in the course of the reaction by a factor of 2–3 (not shown). In accordance, preincubation of the S97A variant with glutaryl-CoA in the absence of an electron acceptor (0.2 mM, 30 min, 4 °C) had a stimulating effect on the initial rate.

The most prominent difference in the active site between decarboxylating and non-decarboxylating seems to be an invariant Tyr in decarboxylating GDHs (Tyr370 in GDH_{Geo}). The dehydrogenation rate of the Y370V variant was about 8% of the wild type rate with a 20-fold higher apparent K_m-value. However, the initial rate of glutaconyl-CoA formation was about 13-fold higher than that of crotonyl-CoA formation. In contrast to the S97A mutant, the initial rate of glutaryl-CoA dehydrogenation decreased in the course of the reaction. This finding might be explained by a higher accumulation of the competitive inhibitor glutaconyl-CoA. After a prolonged incubation, the formation of crotonyl-CoA was again observed. The Y370F variant did not release glutaconyl-CoA and the affinity to glutaryl-CoA was drastically reduced (Table 1).

The apparent V_{max} of the dehydrogenation reaction in S97A+Y370V double mutant was only about 5% of the wild type GDH_{Geo}, K_m was hardly affected. Similar to the Y370V mutant, the rate of glutaconyl-CoA formation was 15-fold higher than crotonyl-CoA formation in the double mutant. Most importantly, glutaconyl-CoA accumulated to a higher extent than with the single amino acid exchange variants. As observed with the S97A and Y370V variants, the glutaconyl-CoA was slowly converted into crotonyl-CoA after prolonged incubation by the residual decarboxylation activity. In both, the S97A+Y370F and the S97V+Y370F/V double mutants, glutaryl-CoA conversion was largely diminished, and no glutaconyl-CoA formation was observed (Table 1).

Table 1
Properties of molecular variants of GDH_{Geo}. Apparent K_m and V_{max} were determined in a spectrophotometric assay; the products crotonyl-CoA and glutaconyl-CoA were determined by HPLC analysis. The numbers represent mean values ± standard deviations.

GDH_{Geo} variants	FAD (mol/mol)[a]	K_m (μM)	V_{max} (μmol mg^{-1} min^{-1})	Glutaconyl-CoA formation
Wild type	0.98	35 ± 12	5.3 ± 0.5	–
E89A	0.79	23 ± 3	0.100 ± 0.003	–
S97A	0.22	9 ± 3.5	0.021 ± 0.001	+
S97V	0.26	n.d.	<0.001	–
Y370F	0.96	4320 ± 2670	0.44 ± 0.25	–
Y370V	1.18	630 ± 140	0.42 ± 0.06	++
S97A+Y370F	0.28	158 ± 64	0.0044 ± 0.0007	–
S97A+Y370V	0.78	27 ± 5	0.25 ± 0.11	+++
S97V+Y370F	0.55	n.d.	<0.001	–
S97V+Y370V	0.50	650 ± 420	0.046 ± 0.020	–

[a] After incubation with 0.5 FAD followed by removal of excess FAD; n.d. = not determined.

4. Discussion

In non-decarboxylating GDHs the tight bidentate complex between the substrate-carboxylate and the Arg-guanidinium was suggested to prevent decarboxylation [14]. In contrast, one oxygen atom of the carboxylate is in a hydrogen bond distance to Ser97 and Tyr370 in decarboxylating GDHs (GDH_{Geo} numbering, [5]). Exchange of the invariant Arg94 in GDH_{hum} was reported to affect V_{max} and K_m; however, no prevention of decarboxylation was reported [4]. In this work, we demonstrated that exchange of Ser97 and Tyr370 did not only affect V_{max} and K_m, but also converted the decarboxylating GDH_{Geo} into a predominantly non-decarboxylating GDH (in case of the Y369V variant). This effect was increased in the double mutant with respect to the amount of glutaconyl-CoA formed.

The prevention of decarboxylation in non-decarboxylating GDH_{Geo} variants can be explained by a tightened binding of the substrate carboxylate in conjunction with a diminished polarization of the C4–C5 bond. The missing electrostatic interactions between the hydroxyl groups of Tyr370 and Ser97 with both the Arg-guanidinium and the substrate-carboxylate in these variants will in turn result in a more rigid electrostatic interaction between the latter two. Such a conversion is expected to require major structural changes in the vicinity of the substrate carboxylate.

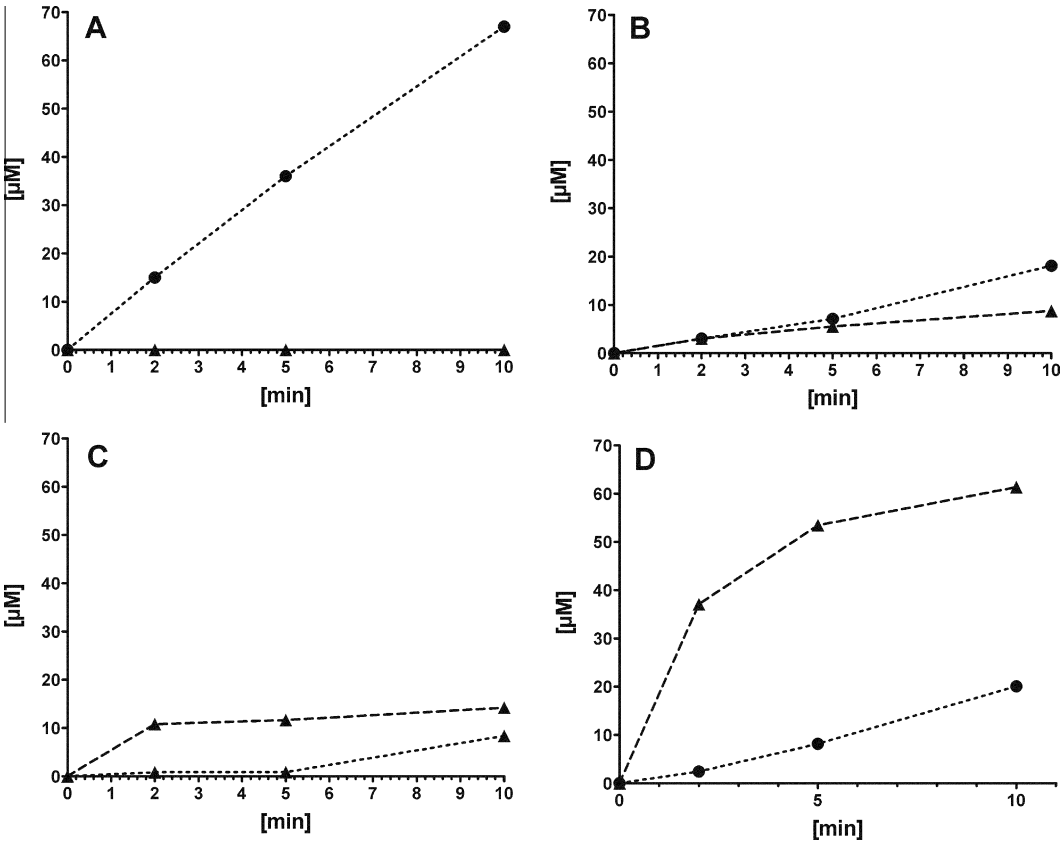

Fig. 2. Time-dependent formation of products from glutaryl-CoA (0.2 mM) in wild type GDH_{Geo} and molecular mutants. ● crotonyl-CoA, ▲ glutaconyl-CoA. (A) Wild type GDH_{Geo}, (B) GDH_{Geo} S97A mutant, (C) Y370V mutant, (D) S97A+Y370V double mutant. Protein concentration in A. was approximately 1/20 of that in B–D.

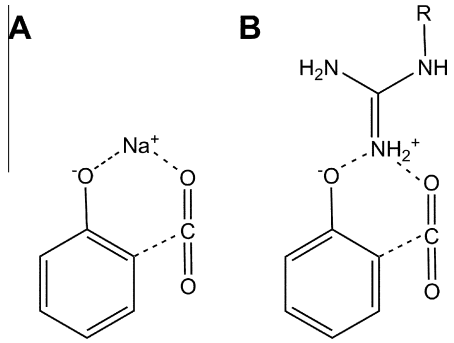

Fig. 3. Proposed intermediates during Kolbe–Schmitt synthesis and the decarboxylation reaction of GDHs. (A) Sodium–phenolate–CO_2-complex in the Kolbe–Schmitt synthesis of hydroxybenzoic acids. (B) Putative guanidinium–tyrosinate–CO_2-complex during the decarboxylation step of GDHs. While under the conditions of the Kolbe–Schmitt synthesis the sodium–phenolate is carboxylated in ortho-position, the CO_2 will be released in decarboxylating GDHs.

Thus, only amino acid exchanges with non-bulky amino acids, such as S97A and Y370V, resulted in active, non-decarboxylating GDHs. Notably, the Y370V exchange is realized in all wild type non-decarboxylating GDHs [12,14].

The tendency for decarboxylation of the glutaconyl-CoA intermediate will generally depend on the leaving group potential of CO_2, which in turn depends on an appropriate CO_2 binding site. In GDH_{hum}, the phenolic hydroxyl group of Tyr369 is in a hydrogen bond distance to the Arg-guanidinium (3.1 Å) and to the substrate carboxylate (3.1 Å, Fig. 1) [14]. Therefore, it is plausible that cleavage of the C4–C5-bond results in the formation of a transient guanidinium–phenolate–CO_2 complex. Such an arrangement shows some analogy to the transition state of the Kolbe–Schmitt synthesis reaction, where a sodium–phenolate is attacked by CO_2 to yield an ortho-hydroxy carboxylic acid ([16–18], Fig. 3). Possibly, the guanidinum-phenolate moiety realized between the Arg and Tyr residues of decarboxylating GDHs finds its counterpart in the sodium phenolate moiety in the Kolbe–Schmitt synthesis. In both cases an attractive binding site for carbon dioxide would be formed. In decarboxylating GDHs, the electrophilicity of the CO_2-leaving group is clearly not sufficient for Tyr-phenolate carboxylation in comparison to the higher temperature and pressure employed in the Kolbe–Schmitt reaction. Biological Kolbe–Schmitt-like reactions have been described for a number of non-oxidative hydroxybenzoate decarboxylases, which show some reversibility at high CO_2 concentrations [19]. An exception is the phenylphosphate carboxylase involved in the anaerobic aromatic catabolism of phenol, which catalyzes the unidirectional carboxylation of the substrate under physiological conditions at the expense of an energy-rich phenol-phosphate-linkage [20,21]. Interestingly, this enzyme is potassium cation-dependent and carboxylates the phenolate substrate in para-position as expected in the chemical Kolbe–Schmitt synthesis [22].

The recently discovered crotonyl-CoA carboxylase/reductase, an enoyl-CoA carboxylating reductase, shows some analogy to decarboxylating GDHs [23]. It is proposed to have evolved from a butyryl-CoA dehydrogenase by a few amino acid exchanges resulting in a preference for carboxylation rather than protonation of the thioester enolate intermediate.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.03.063.

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