FEBS Letters 585 (2011) 1317–1321

FEBS Letters

journal homepage: www.FEBSLetters.org

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

Jörg Schaarschmidt, Simon Wischgoll, Hans-Jörg Hofmann, Matthias Boll *

Institute of Biochemistry, University of Leipzig, Germany

ARTICLE INFO

Article history:
Received 17 March 2011
Revised 30 March 2011
Accepted 31 March 2011
Available online 6 April 2011

Edited by Stuart Ferguson

Keywords:
Glutaryl-CoA
Acyl-CoA dehydrogenase
Decarboxylase
Kolbe–Schmitt synthesis
Anaerobic aromatic metabolism
Geobacter

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

* Corresponding author. Address: Institute of Biochemistry, Brüderstr. 34, D-04103 Leipzig, Germany. Fax: +49 341 9736910.
E-mail address: boll@uni-leipzig.de (M. Boll).

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doi:10.1016/j.febslet.2011.03.063

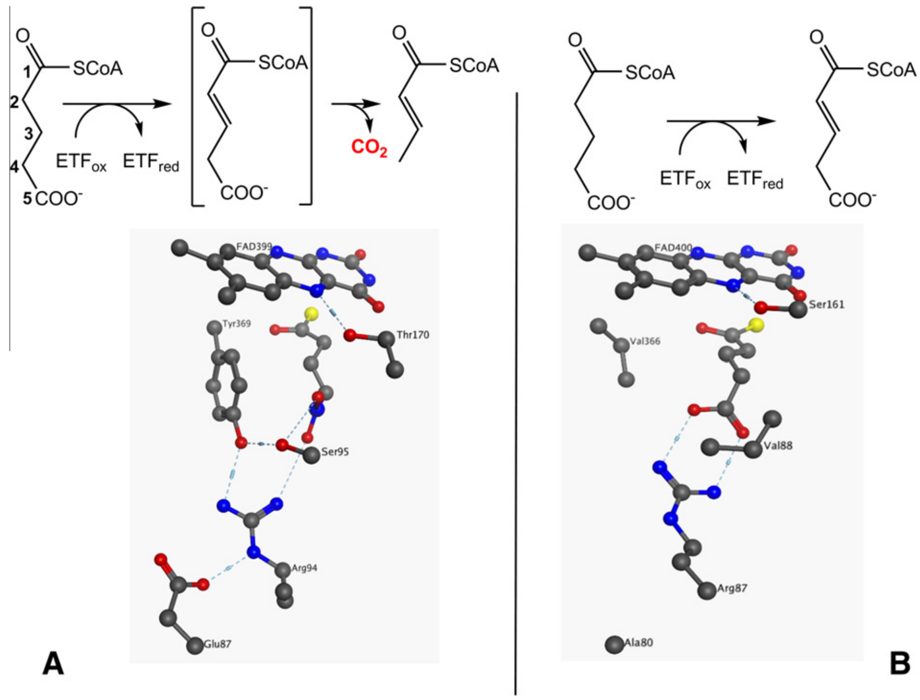

Fig. 1. Reactions catalyzed and amino acid residues involved in substrate carboxylate binding in decarboxylating GDH_{hum} and non-decarboxylating GDH_{Des}. (A) Reaction and active site of GDH_{hum} with bound 4-nitrobut-2-enoyl-CoA; the glutaconyl-CoA intermediate (in brackets) is not released during 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