Identification of glutamate β54 as the covalent-catalytic residue in the active site of glutaconate CoA-transferase from *Acidaminococcus fermentans*

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Abstract In the course of glutamate fermentation by *Acidaminococcus fermentans* glutaconate coenzyme A-transferase catalyzes the transfer of CoAS⁻ from acetyl-CoA to (*R*)-2-hydroxyglutarate, forming (*R*)-2-hydroxyglutaryl-CoA. Glutamate (E) 54 of the β-subunit was postulated to be directly involved in catalysis by formation of a CoASH ester intermediate [(1994) Eur. J. Biochem., in press]. In order to prove this preliminary result, the following mutations, βE54A, βE64A, βE54Q and βE54D, were introduced by mismatch oligonucleotide priming. As expected, βE54A was inactive (0.02% of the wild-type), whereas βE64A and βE54D were active, 30% and >7%, respectively. However, no CoASH intermediate was detected in the latter mutant, indicating a change in the catalytic mechanism. The activity of the βE54Q mutant increased from 1% to almost 100% upon incubation with acetyl-CoA and glutaconate at 37°C within 40 h. Hence, the substrates induced the conversion of the mutant glutamine residue into the glutamate residue of the wild-type enzyme.

Key words: Glutaconate CoA-transferase; Site-directed mutagenesis; βE54 as covalent catalytic residue; Post-translational activation by the substrates; *Acidaminococcus fermentans*

1. Introduction

Glutaconate CoA-transferase (Gct; EC 2.8.3.12) from the strict anaerobic bacterium *Acidaminococcus fermentans* [1] participates in the fermentation of glutamate via the hydroxyglutarate pathway [2,3]. The enzyme is responsible for the transfer of the CoAS⁻ moiety from acetyl-CoA to the characteristic intermediate of this pathway, (*R*)-2-hydroxyglutarate to give (*R*)-2-hydroxyglutaryl-CoA, thus activating the dicarboxylic acid for further metabolization. The enzyme consists of two subunits, GctA and GctB, with molecular masses of 36 and 29 kDa, respectively, and has a heterooctameric structure, $\alpha_4\beta_4$. Glutaconate CoA-transferase accepts a variety of substrates, with glutaconate and glutaconyl-CoA being the best [1].

In the case of the pig heart 3-oxoacid CoA-transferase (EC 2.8.3.5) Jencks and co-workers [4] were able to show that the γ-carboxyl group of a specific glutamyl-residue of the enzyme is directly involved in catalysis by reversibly forming a CoASH ester. Parales and Harwood [5] postulated, by sequence alignment of four CoA-transferases, that this glutamyl residue (E344) is part of the short consensus sequence SENG, which was tentatively confirmed by Rochet and Bridger [6]. Recently the genes of glutaconate CoA-transferase (*gctA* and *B*) have been cloned, sequenced and over-expressed in *E. coli* [3]. This enzyme, however, did not show any significant sequence similaritiy to the other CoA-transferases; especially the consensus SENG was not detected.

Mack et al. [3] labelled the enzyme by reducing the CoASH ester intermediate with $NaB[^3H]_4$, digested the resulting enzymatically inactive protein with trypsin and isolated the radioactive peptide. Edman degradation showed that βE54 was converted into a radioactive compound different from the phenylthiohydantoin-derivative of glutamate. Hence, E in the sequence HIIVES was probably reduced to 2-amino-5-hydroxy- [5-^{3}H]valerate. In this paper we describe site-directed mutagenesis, labelling and activation experiments which further support this finding.

2. Materials and methods

2.1. Bacterial strains, phages and plasmids

See [3,7,8] for a description. Bacteria were grown at 37°C. The liquid and solid media for *E. coli* DH5α were standard nutrient broth (Merck); for *E. coli* XL1-Blue, the double concentrated yeast extract medium (2 × YT, [7]) was used.

2.2. Site-directed mutagenesis

The 1.7 kb *Eco*RI–*Sal*I fragment from plasmid pMM2 [3] containing the two genes of Gct (*gctA* and *B*) was subcloned into pBluescript SK(+) (Stratagene) and transformed into *E. coli* XL1-Blue. A growing culture of this resulting clone was infected with the helper phage VCSM13 (Stratagene). The single-stranded DNA was recovered according to the Stratagene protocol. Site-directed mutagenesis was performed on single-stranded DNA, using the method of Eckstein's group [9]. The following mismatch oligonucleotide primers were used: Mut2996, 5'-CATCGTGGCAAGCGGTCTG-3' to change βE54 to A; Mut3026, 5'-CCGGTGGCAGTTCCCCG-3' to change βE64 to A; MutGln 5'-CATCGTGCAAAGCGGTCTG-3' to change βE54 to Q; MutAsp 5-'CATCGTGGATAGCGGTCTG-3' to change βE54 to D. Sequence analysis of the mutant regions was performed by the dideoxy chain termination method [10] using the Sequenase Version 2.0 DNA Sequencing Kit from United States Biochemical Corp. (Cleveland, USA).

2.3. DNA manipulations

DNA manipulations were performed by standard procedures [7]. The mutant genes in pBluescript SK(+) were digested by *Eco*RI/*Sal*I and the resulting 1.7 kb fragment (*gctA* and B^{mut}) was subcloned into the expression vector pJF118HE [8].

2.4. Enzyme assay and ^{3}H-labelling

The activity of glutaconate CoA-transferase and of the various mutant enzymes was measured as described elsewhere [1]. Cells were grown in 100 ml Erlenmeyer-flasks and induced with 300 μM isopropyl-1-thio-β-D-galactoside when reaching $A_{578} = 2$; at $A_{578} = 5.5$ the cells were

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harvested. Crude extracts of *E. coli* strains containing the wild-type and mutant enzymes were directly used in the assay. The different enzyme species were labelled in the crude extract with $NaB[^3H]_4$ as described by Buckel et al. [1]. Protein was determined using the Bio-Rad assay (Bio-Rad, Munich).

3. Results

Site-directed mutagenesis yielded the following changes of the wild-type enzyme, βE54A, βE64A, βE54Q and βE54D, as confirmed by DNA sequencing. SDS-PAGE of crude extracts of the resulting strains showed that the genes of the mutant enzymes (*gctA* and B^{mut}) were expressed at a similar level as the wild-type genes. Two fat bands appeared in each lane of the gel at about 30 and 36 kDa, representing GctA and $GctB^{mut}$, respectively (Fig. 1A). The *E. coli* strain producing the mutant βE54D showed a lower growth rate and the amount of the recombinant enzyme was reduced (Fig. 1A, lane D).

As expected, βE54A hardly showed any activity, supporting the view that indeed βE54 is the active site residue (Table 1). Introducing the same change at another glutamate residue, ten amino acid residues apart (βE64A), yielded a mutant which still was active (30%). In the mutant βE54D the negative charge was retained. This enzyme species also showed activity (7%), which, due to its lower amount, even could be twice as high. The βE54Q mutant had a low but significant activity (1%, see below).

In order to detect the enzyme CoA thiol ester intermediate, the mutant enzymes were incubated with tritiated sodium borohydride in the presence of glutaryl-CoA. Under these conditions the wild-type enzyme and βE54Q were completely inactivated, whereas βE64A still contained 33% of its original activity (Table 2). The residual activities of βE54A and βE54D remained unchanged. These results were corroborated by incorporation of radioactivity only into those enzyme species which were inactivated by $NaBH_4$ (Fig. 1B). The radioactivity of βE54Q was probably too low (1%) to be visualized.

The mutant βE54Q was tested again after 5 weeks of storage in the cold room (4°C). During this period the specific activity increased approximately twofold from 0.59 U/mg protein to 1.3 U/mg protein. The enzyme was then incubated in the presence of acetyl-CoA (0.39 mM) and glutaconate (10 mM) at 37°C. At the times indicated samples of this incubation mixture were tested for activity (Fig. 2). After 40 h the specific activity of the mutant βE54Q (23 U/mg) rose to close to that of the wild-type enzyme (26 U/mg protein). Curiously, the wild-type enzyme was transiently activated, irrespective of whether the substrates were present or absent.

Table 1
Specific activities of the mutant enzymes in comparison to the wild-type enzyme

Enzyme species	Specific activity (U/mg)	Specific activity relative to that of the wild-type enzyme (%)
Wild-type	61	100
βE54A	0.012	0.02
βE64A	16	30
βE54D	4.0	7
βE54Q	0.58	1

Crude extracts of *E. coli* strains carrying *gctA* and B^{mut} on pJF118HE were directly assayed for activity.

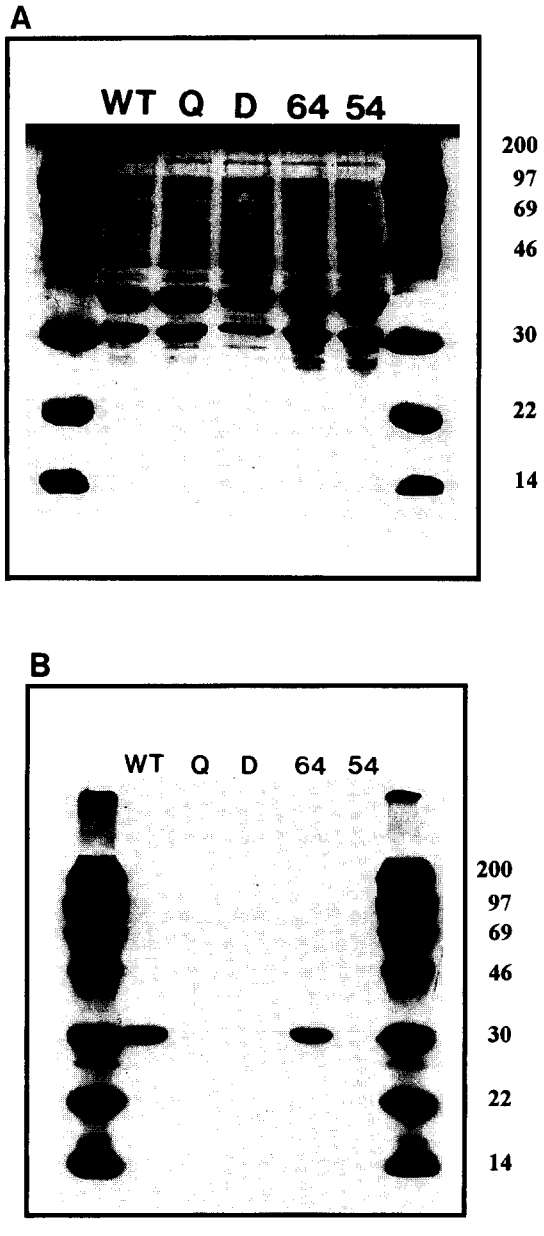

Fig. 1. SDS-PAGE of crude extracts of the mutant *E. coli* strains containing *gctA* and B^{mut} in pJF118HE after treatment with $NaB[^3H]_4$ in the presence of glutaryl-CoA. To each lane the same amount of protein (10 μg) was applied. WT, wild-type; Q, βE54Q; D, βE54D; 64, βE64A; 54, βE54A. (A) The gel was stained with Coomassie. The two fat bands correspond to GctA (36 kDa) and B (29 kDa) of the wild-type and mutant enzymes (GctA and $GctB^{mut}$), respectively. The sizes of the protein standards are indicated to the right (kDa). (B) Scintigram of the dried gel. The single bands in the lanes WT and 64 represent the ^3H-labelled β-subunit.

4. Discussion

All the data together clearly indicate that βE54 is indeed the active site glutamate residue. The βE54A mutant hardly showed any activity and did not become labelled (Table 1, Fig.

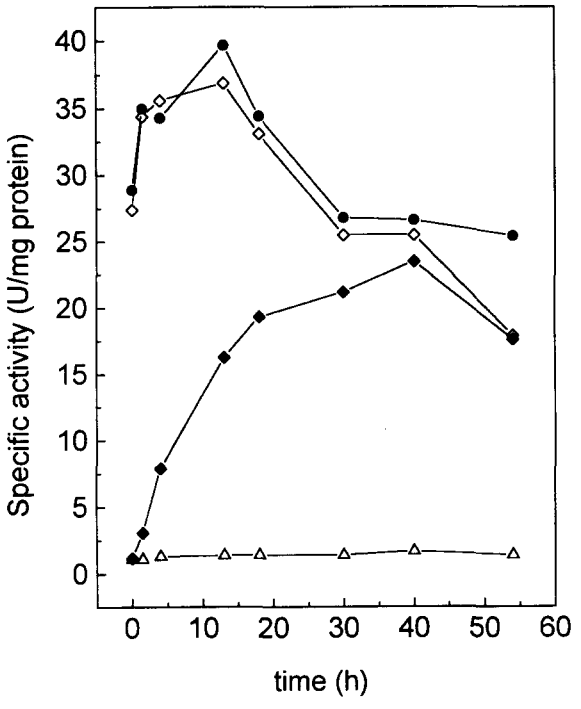

Fig. 2. Activation of the glutamine mutant. The mutant enzyme βE54Q (◆) was incubated in the presence of the substrates acetyl-CoA (0.39 mM) and glutaconate (10 mM) at 37°C. As a control, βE54Q was incubated in the absence of the two substrates (△). The wild-type enzyme was treated in the same way and was incubated in the presence (●) and absence (◇) of the substrates.

1B). In contrast, the mutant βE64A was still active and became labelled. Hence, CoA, as in the wild-type enzyme, is still bound to βE54 during catalysis. Slight conformational changes, however, are introduced by replacing βE64 by A, resulting in a remarkable loss of activity (Table 1) and less effective inactivation (Table 2). βE54D is a conservative exchange, probably not dramatically affecting the overall conformation of glutaconate CoA-transferase. A very interesting fact is that this mutant did not become ^{3}H-labelled at all (Fig. 1B), showing again that the crucial amino acid residue involved in catalysis is affected. The

Table 2
Inactivation experiments

Enzyme species	Specific activity before adding NaB[^{3}H]$_4$ (U/mg protein)	Specific activity after adding NaB[^{3}H]$_4$ (U/mg)	Inactivation (%)
Wild-type	61	2.1	97
βE54A	0.012	0.012	0
βE64A	16	5.4	66
βE54D	4.0	3.9	3
βE54Q	0.58	0.0074	99

Crude extracts of *E. coli* strains carrying *gctA* and B^{mut} on pJF118HE were directly assayed for glutaconate CoA-transferase activity in the presence of the substrate glutaconyl-CoA before and after adding NaB[^{3}H]$_4$.

enzyme is obviously still able to catalyze the transfer of CoA but not by transiently forming a thiol ester between CoASH and βD54. Thus, this mutant protein must exhibit its catalytic activity in a different way by simply bringing the two substrates in to proximity with each other. Examples for CoA-transferases without thiol ester intermediates are the α-subunits of citrate lyase (EC 4.1.3.34) [11,12] and citramalate lyase (EC 4.1.3.25) [13].

Another conservative mutation with respect to size is achieved by changing βE54 into Q. This mutant of course is not able to form a thiol ester bond and thus should be inactive. However, βE54Q shows about 1% activity which can be completely removed by $NaBH_4$, like in the wild-type enzyme. Furthermore, incubation in the presence of the substrates regenerates the enzymatic activity. The results strongly suggest that β54Q of the mutant is converted to the wild-type βE54 (Fig. 3). The reaction requires both acetyl-CoA and glutaconate. No significant activation was observed by omitting one of them or replacing acetyl-CoA by CoASH. The mechanism of this activation could be analogous to that proposed for the second half reaction of the CoA-transfer [13]. Hence, NH_2^- rather than $CoAS^-$ is transferred whereby also a mixed anhydride should be formed as intermediate. The bad nucleofugicity of NH_2^- may explain the slow activation (40 h at 37°C). Jencks proposed that the energy released by binding the CoA moiety of specific substrates to the enzyme is used to lower the energy of the

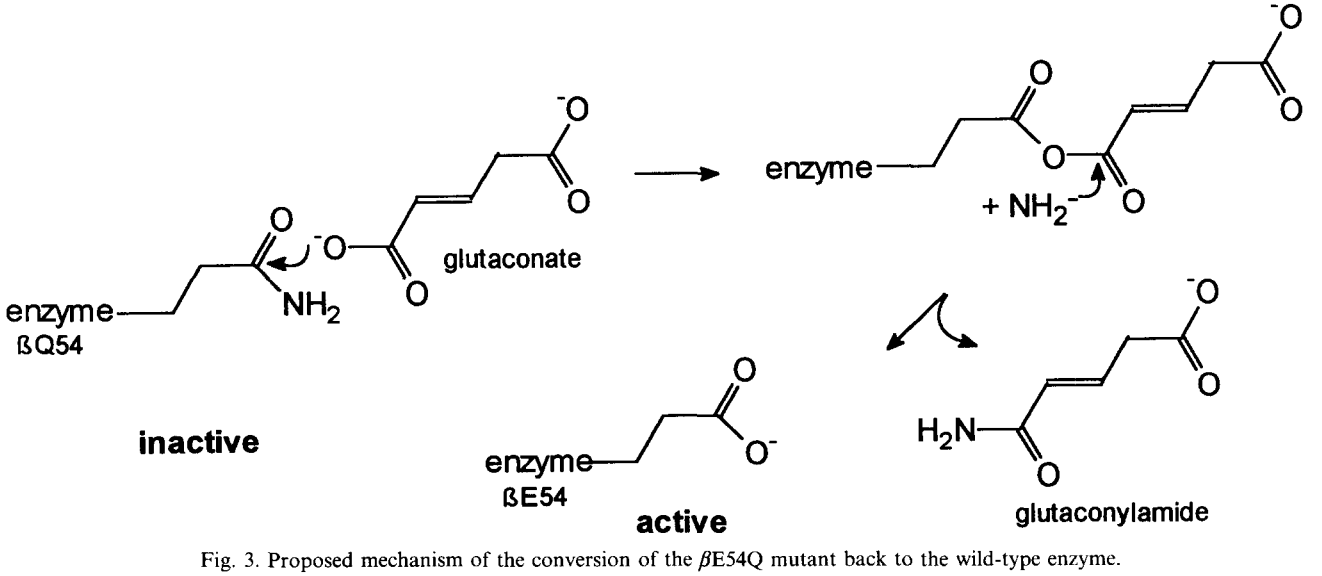

Fig. 3. Proposed mechanism of the conversion of the βE54Q mutant back to the wild-type enzyme.

transition state and thus enhance the rate of catalysis [14]. Acetyl-CoA might have a similar function during the activation of the βE54Q mutant.

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