The masked cysteine residues in methylmalonyl-CoA mutase from *Propionibacterium shermanii* are essential for catalytic activity

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Abstract Two masked cysteine residues have been reported in methylmalonyl-CoA mutase from *Propionibacterium shermanii*, Cys-535 in the α-subunit and Cys-517 in the β-subunit, which are revealed only after reduction of the denatured enzyme with dithiothreitol. It has been postulated that these residues are involved in disulphide linkages to unknown thiols of low M_r. These two masked cysteine residues have been changed to an alanine, individually. Both the mutants, C535αA and C517βA, were inactive. This shows that both these residues are essential for catalytic activity.

Key words: Masked cysteine residue; Methylmalonyl-CoA mutase

1. Introduction

Methylmalonyl-CoA mutase (EC 5.4.99.2) (MCM) is an adenosylcobalamin-dependent enzyme that catalyses the interconversion of succinyl-CoA (3-carboxypropionyl-CoA) and (2*R*)-methylmalonyl-CoA, both in mammalian liver [1] and in the propionate-producing fermentation of certain bacteria such as *Propionibacterium shermanii* [2,3].

MCM from *P. shermanii* is a dimer consisting of an α-subunit (M_r 79 000) and a β-subunit (M_r 67 000) [4]. The structural genes have been cloned and sequenced [5], and co-expressed in *Escherichia coli* [6]. The apoenzyme purified from the recombinant *E. coli* can be converted into active holoenzyme by addition of adenosylcobalamin [6]. The amino acid sequence for *P. shermanii* MCM deduced from the sequence of the structural genes shows that the β-subunit contains four, and the α-subunit contains two cysteine residues. However, treatment of the denatured enzyme with iodo[^{14}C]acetic acid resulted in only four moles of ^{14}C label being incorporated per mole of mutase [7]. Pretreatment of the denatured enzyme with dithiothreitol before treatment with iodo[^{14}C]acetic acid resulted in six moles of ^{14}C label being incorporated per mole of mutase. SDS/polyacrylamide-gel electrophoresis in the presence and absence of dithiothreitol shows no inter-subunit disulphide bond is present in the denatured enzyme. This ruled out the possibility of the masked thiols being involved in inter-subunit disulphide linkage. Also, hydroxylamine treatment was not able to unmask the thiol groups, showing that these residues were not involved in thioester linkages either. These observations indicated that the masked thiols were involved in disulphide linkages with thiols of low M_r. Evidence was also obtained that a volatile thiol was released by dithiothreitol treatment which was trapped using $HgCl_2$. The masked thiols were found to be located at Cys-535(α) and Cys-517(β) [7]. Furthermore, these thiols were found to occupy exactly equivalent positions in the mutually homologous α- and β-subunits. This further indicates that these residues have similar environments in the native protein [7].

Recently the crystal structure of the holoenzyme along with desulphoCoA has been determined at 2Å resolution [8]. In the structure it is found that these thiols are part of the linker region which encloses the N-terminal barrel domain and connects it to the C-terminal domain in each subunit. The residue Cys-535 in the α-subunit is however not found in the described active site, though the present structure lacks both the adenosyl group of the cofactor and the rearranged part of the substrate. Hence, a detailed description of the active site is awaited for which further structures of substrate and inhibitor complexes are required.

This work describes the site-directed mutagenesis of these masked thiols and the effect of these changes on the enzyme.

2. Materials and methods

2.1. Plasmids, phage and oligonucleotides

The pUC18 and pUC19 vectors, pUC118 and pUC119 vectors, the phagemid pTZ18R, and the M13mp18 and M13mp19 sequencing vectors were obtained from Pharmacia (UK) Ltd. The dual plasmid pT7-7 expression system was the kind gift of Dr. S. Tabor, Harvard Medical School. Helper phage VCSM13 was supplied by Stratagene Inc. (San Diego, CA, USA) as part of their 'Bluescript' cloning and sequencing system. The pTZ18R-based double mutant plasmid pM7mut3, containing the *P. shermanii* MCM genes with an *Nde*I site at the start codon for the upstream β-subunit, and with the start codon for the α-subunit altered from GTG to ATG, has been described previously [6].

The oligonucleotides used for site-directed mutagenesis and sequencing were synthesised on a Millipore Biosearch Cyclone automated synthesiser by Mike Weldon of the Wellcome Trust-funded oligonucleotide synthesis service in the Department of Biochemistry, University of Cambridge.

2.2. Bacterial strains

E. coli TG1*recO* [9] was from Dr. P. Oliver, Department of Genetics, University of Cambridge. *E. coli* K38 [K12, HfrC, (λ)] containing the plasmid pGP1-2 was from Dr S. Tabor, Harvard Medical School. The pGP1-2 plasmid contains a heat-inducible gene for T7 RNA polymerase [10]. The *E. coli* NM538 [supF, hsdR, trpR, lacY] [11] was from Dr D.M. Carrington, Department of Biochemistry, University of Cambridge. *E. coli* TG1*recO* was stored on M9 minimal medium agar plates [12] to maintain selection for the F′ episome. The other *E. coli* strains were grown or maintained on 2TY agar plates or liquid medium with antibiotic supplements as required for plasmid selection. Media constituents were from either Oxoid (Basingstoke, UK) or Lab M (Bury, UK). Yeast extract was from Beta-lab (East Molesey, UK).

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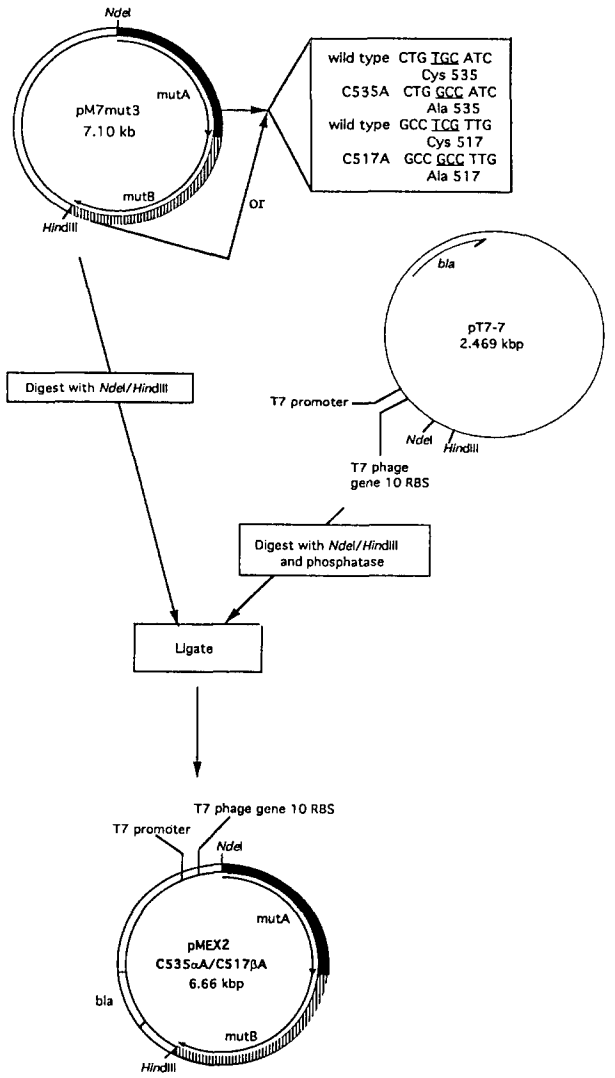

Fig. 1. Construction of the overexpression plasmid for the C535αA and C517βA mutants.

2.3. Site-directed mutagenesis

The phagemid pM7mut3 [6] was used for in vitro mutagenesis, using the phosphorothioate method [13]. Single-stranded DNA was produced by superinfection of cells containing pM7mut3 with the helper phage VCSM13. The oligonucleotide designed to achieve the mutation of Cys-517 (β) to alanine was the 20-mer: 5′-TTCCTGGCC**GCC**TTGGGCAC-3′ and that of Cys-535 (α) to alanine was the 20-mer: 5′-CTGAAGCTGG**CC**ATCGACGC-3′. These were purified by gel electrophoresis, and 5′-phosphorylated. These oligonucleotides (5 pmole) were next annealed to 5 μg template by heating for 3 min to 70°C followed by slow cooling to room temperature. The mutagenesis was carried out using the Amersham in vitro mutagenesis kit (version 2) according to the supplier's instructions. The mutant homoduplexes synthesised were used to transform *E. coli* TG1*recO* to ampicillin resistance. For each mutant, phagemid was recovered from eight recombinant colonies and characterised by DNA sequencing using the dideoxy chain termination method, to show that the desired change had been accomplished. One of each mutant phagemid was selected for further work, and designated as pMC517A and pMC535A (Fig. 1). Subsequently, a set of primers was used to re-sequence in their entirety the structural genes for both MCM structural genes. No additional unwanted alterations were found.

2.4. High level expression of the mutant MCM genes

The plasmids pMC517A and pMC535A were digested with *Nde*I and *Hin*dIII and the resulting *Nde*I-*Hin*dIII fragment were gel-purified and ligated into appropriately digested, phosphatase-treated pT7-7 [6,10] and the ligation mixture was transformed into *E. coli* TG1*recO*. Heat shock during transformation was done at 30°C for 5 min, to avoid induction of MCM expression. Ampicillin-resistant colonies were picked and screened for the presence of the plasmid containing the *Nde*I-*Hin*dIII fragment. These expression plasmids, termed pMEX2 C517βA and pMEX2 C535αA (Fig. 1), were transformed into *E. coli* K38/pGP1-2 [6,10] for expression of the mutant mutase genes, and transformants were selected after overnight growth at 30°C on 2TY agar plates supplemented with ampicillin (100 μg/ml) and kanamycin (60 μg/ml). For large-scale growth of the strains containing the expression plasmid, five 2-litre flasks, each containing 600 ml of 2TY medium (100 μg/ml ampicillin, 60 μg/ml kanamycin) were inoculated with an overnight culture (3.5 ml) and incubated at 38°C until the A_{600} reached 1.5. Fresh 2TY medium (400 ml), pre-heated to 65°C, was then added to each flask which was shaken for 1 min. The flasks were then chilled quickly on ice and incubated for a further 2.5 h at 28°C. The cells were harvested by centrifugation and the paste was stored at −70°C.

2.5. Partial purification of the mutant proteins

Partial purification of the mutant proteins was carried out using ion exchange chromatography. The cells were broken by sonication using a tip sonicator operated at 90% of full power output, in cold 100 mM Tris-HCl pH 7.5 containing 5 mM benzamidine hydrochloride, 0.5 mM phenylmethanesulphonyl fluoride and 2 mM dithiothreitol. The sonication was done in bursts of 2 min, at intervals of 10 min with cooling on ice, until a clearing of the protein solution was observed. Finally the cell debris was removed by centrifugation at $12000 \times g$ at 4°C for 30 min. The supernatant was diluted with an equal volume of water and was loaded onto a 300 ml Q-Sepharose column. A 1-litre gradient of 0–0.6 M KCl in 50 mM Tris-HCl buffer pH 7.5 containing 0.2 mM DTT and a flow rate of 180 ml/h was used to elute the mutant mutases. None of the mutants had any detectable activity.

2.6. Measurement of MCM activity

Measurements of MCM activity were carried out at 30°C and pH 7.5 using a continuous spectrophotometric assay in which the production of (2*R*)-methylmalonyl-CoA is coupled to NADH oxidation by malate dehydrogenase [6,14].

3. Results and discussion

We have changed both the masked thiol residues into ala-

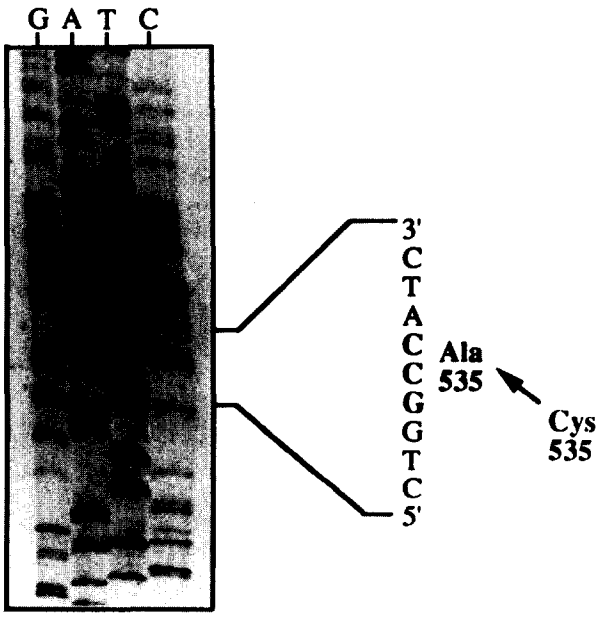

Fig. 2. DNA sequencing gel showing the cysteine to alanine substitution brought about in the C535αA mutant.

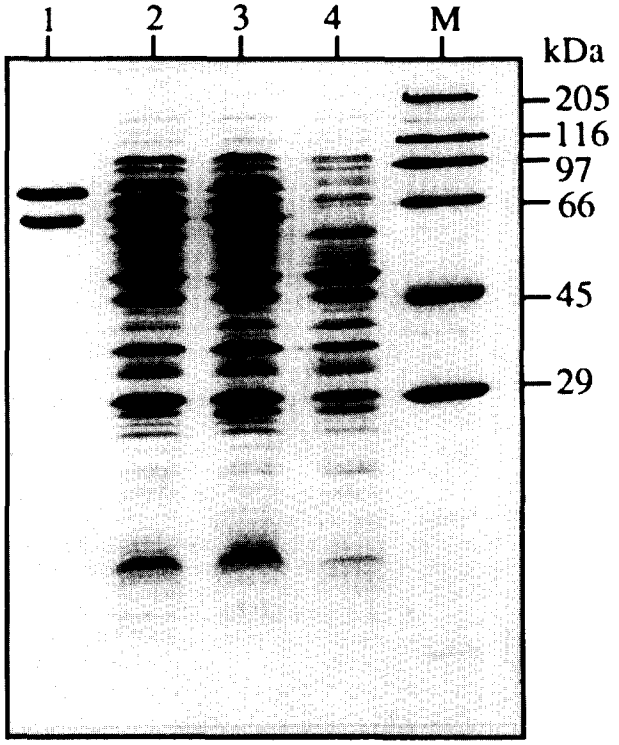

Fig. 3. A Coomassie blue-stained 15% SDS polyacrylamide gel showing the overexpression of the C535αA mutant. 1. Purified wild type *P. shermanii* MCM. 2. Whole cell extract of *E. coli* K38/pGP1-2 containing pMEX2 C535αA, induced. 3. Soluble fraction of the whole cell extract of *E. coli* K38/pGP1-2 containing pMEX2 C535αA, induced. 4. Insoluble fraction of the whole cell extract of *E. coli* K38/pGP1-2 containing pMEX2 C535αA, induced. M: High molecular weight markers.

nine, individually (Fig. 2 shows the cysteine to alanine substitution brought about in C535αA). Both these mutant proteins have been successfully overexpressed using the pT7-7/pGP1-2 dual plasmid expression system [10] (Fig. 3 shows the overexpression of the C535αA mutant). This indicates that both the mutants are stably folded.

Partial purification of the mutant enzymes was carried out to remove the NADH oxidase activity in the presence of which the coupled assay [14] cannot be used. Both the mutant proteins were successfully purified so as to remove the undesirable NADH oxidase activity. Activity measurements on these partially purified enzymes indicated that in both the mutants, enzyme activity had been abolished. These masked thiol residues form a part of the linker region. The residue in the α-subunit, Cys-535, is not involved in cobalamin binding or in substrate binding. It is also not necessarily directly involved in the formation of the active site. Hence the total loss of activity on its substitution with alanine indicates that it plays an extremely important role in the proper folding of the active-site region. Also, the β-subunit has no obvious major function although it contributes one residue to the substrate binding site. However, it has been observed that the α-subunit when expressed on its own is unable to fold (Dr. M. Patchett and Dr. N. McKie, personal communication). Hence, the β-subunit definitely plays a major role in the stable folding of the α-subunit. The total loss of activity on the substitution of the Cys-517 residue indicates that it plays a very important role in the correct folding of the β-subunit and hence the α-subunit. However, the absolute structural role of these thiols will have to be confirmed using the structure of MCM containing the intact substrate molecule. In conclusion, this work shows that the masked thiols play an essential role in the catalysis of *P. shermanii* MCM by direct or indirect involvement in the active site formation (Cys-535α) and the correct folding of the α-β heterodimer (Cys-517β).

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