Homocysteine biosynthesis pathways of *Streptococcus anginosus*

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Received 28 January 2003; received in revised form 5 March 2003; accepted 11 March 2003

First published online 1 April 2003

Abstract

A gene (*cgs*) encoding cystathionine γ-synthase was cloned from *Streptococcus anginosus*, and its protein was purified and characterized. The *cgs* gene and the immediately downstream *lcd* gene were shown to be cotranscribed as an operon. High-performance liquid chromatography analyses showed that the *S. anginosus* Cgs not only has cystathionine γ-synthase activity, but also expresses O-acetylhomoserine sulfhydrylase activity. These results suggest that *S. anginosus* has the capacity to utilize both the transsulfuration and direct sulfhydrylation pathways for homocysteine biosynthesis.

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Keywords: Methionine; Homocysteine; Cystathionine synthase; Transsulfuration pathway; Direct sulfhydrylation pathway

1. Introduction

Microorganisms can synthesize the sulfur-containing amino acids L-cysteine and L-methionine from inorganic sulfate, whereas non-ruminant animals require a dietary source of L-methionine or L-homocysteine [1]. There are two alternative pathways for L-homocysteine biosynthesis: the transsulfuration pathway in which the sulfur atom is transferred from L-cysteine to L-homocysteine via a thioether intermediate, L-cystathionine, and the direct sulfhydrylation pathway where hydrogen sulfide is substituted for L-cysteine (Fig. 1). The transsulfuration pathway is catalyzed by cystathionine γ-synthase and cystathionine β-lyase, while the direct sulfhydrylation pathway is catalyzed by acylhomoserine sulfhydrylase. Finally, methionine is synthesized from homocysteine by homocysteine methyltransferase [1]. Microorganisms such as *Escherichia coli* [2] and some plants [3] use the transsulfuration pathway, while organisms such as *Saccharomyces cerevisiae* [4], *Rhizobium etli* [5], *Pseudomonas aeruginosa* [6], and *Lep- tospira meyeri* [7] utilize the direct sulfhydrylation pathway. *Corynebacterium glutamicum*, yeast, fungi, and some green plants are reported to possess both functional pathways [8–11].

Cystathionine γ-synthase reacts with an activated form of L-homoserine: O-acetyl-L-homoserine (fungi and some bacteria) [12,13], O-succinyl-L-homoserine (enteric and some other bacteria) [14,15], or O-phospho-L-homoserine (plants) [16]. As described above, the pathways for sulfur amino acid biosynthesis are well documented in a few bacteria and eukaryotic cells, while information for many other bacteria is still limited. In streptococcal species, there are no data available on the catabolism of sulfur-containing amino acids, except for our recent papers [17,18]. In this study, we cloned the gene encoding cystathionine γ-synthase in *Streptococcus anginosus*, and characterized its purified product in order to provide evidence that *S. anginosus* possesses the functional transsulfuration and direct sulfhydrylation pathways.

2. Materials and methods

2.1. Bacterial strains and culture conditions

*S. anginosus* FW73 was grown in brain heart infusion (Difco Laboratories) broth at 37°C in a 5% CO₂ atmosphere. *E. coli* BL21 (F⁻ ompT lon) hsdS₂(rK-mK) gal dcm (DE3) obtained from Novagen was grown aerobically in 2×TY broth. When required, the medium was supplemented with 100 μg ml⁻¹ ampicillin.

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2.2. General procedures

Routine molecular biology procedures were carried out as described by Sambrook et al. [19]. Chromosomal DNA of S. anginosus was prepared as described previously [20]. Total RNA was prepared from S. anginosus using the FastPrep device (Savant Instruments) in combination with FastRNA kit-BLUE (BIO 101), as previously described [21].

2.3. Cloning of the S. anginosus cgs gene

Inverse polymerase chain reaction (PCR) [22] was used to isolate the cgs gene located upstream from lcd [17]. Genomic DNA was digested with MspI, and a diluted DNA sample was self-ligated. Samples from the ligation reaction were used as template with relevant primers (forward primer, 5'-TTGTCACTCGTCATAAGAGC-3'); reverse primer, 5'-TTATTGCGGCAAACAATGCA-3'). Under standard PCR conditions. The primers were designed from the sequence in the region upstream from the lcd gene (see Fig. 2A) [17]. The resulting amplicons were cloned into pGEM-T Easy Vector (Promega) and sequenced with an ABI 310 Genetic Analyzer (Applied Biosystems).

2.4. Detecting cgs transcription by reverse transcriptase-mediated (RT)-PCR

RT-PCR analysis was performed by the method of Shibata et al. [21]. Before using the solution for RT-PCR, contaminating DNA was eliminated by digestion with RNase-free DNase. Reverse transcription was performed after removing the DNAse using polymerase–chloroform extraction. Relevant primers for RT-PCR were designed from the sequences of open reading frame 1 (ORF1), cgs, and lcd (see Fig. 2). The oligonucleotide primer pairs consisted of RT-F1, 5'-CCGGCTAATATGCTGAAAT-3'; RT-R1, 5'-TTAATGAAATGAAATTTTGGACG-3'; RT-F2, 5'-GTACCAACACATTCGTCAG-3'; RT-R2, 5'-CAAGCAATTCCTCTTCCGTA-3'; RT-F3, 5'-ATGGATAAAAAGTTACAATTAGA-3'; RT-R3, 5'-TTATACTTCCCAAGCAAATTTT-3'; RT-F4, 5'-CGTTCTGGCTGCTTATCA-3'; RT-R4, 5'-CTGCTCTGCTTAC-3'. Each reverse primer for RT-PCR analysis was used to synthesize cDNA from specific mRNA in total RNA. Reaction mixtures were used as negative controls contained no reverse transcriptase.

2.5. Recombinant protein purification

The recombinant cystathionine $\gamma$-synthase of S. anginosus was purified using the expression vector pGEX-6P-1 (Amersham Biosciences), as previously described [17]. To construct a pGEX-6P-1 derivative designated pCB100, the cgs gene was amplified by PCR (forward primer, 5'-TCCGGATCCGATCAAAAGTTACAAATTTGA-3'; reverse primer, 5'-GAGCTCAGATCTTATACCTGCAAGCAATTT-3'). These primers were designed so that BamHI and SalI restriction sites (underlined) were created in the PCR product. The amplified fragment containing the ORF for the cgs product was digested with BamHI and SalI sites, and then cloned in-frame with glutathione S-transferase (GST, Amersham Biosciences) in the pGEX6P-1 vector. The E. coli transformant was grown at 37°C until an optical density at 550 nm of about 1.0 was attained. Expression of cgs was induced with 0.1 mM isopropyl-$\beta$-thiogalactopyranoside. The cells were harvested 1.5 h after induction and lysed by ultrasonication. The cell extract was obtained by centrifugation at 18000×g for 30 min at 4°C. The Cgs protein was purified using a MicroSpin GST Purification Module (Amersham), as previously described [17]. Briefly, the GST-fusion protein was purified by affinity chromatography with glutathione—Sepharose 4B medium and cleaved with PreScission protease. The purity of the protein was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

2.6. Enzyme assay for cystathionine $\gamma$-synthase

Cystathionine $\gamma$-synthase activity was determined by the method of Ravel et al. [23], which measures the disappearance of $\gamma$-cysteine in the ninhydrin reaction. Briefly, the assay was carried out with 100 μl of 20 mM MOPS–NaOH (pH 7.5) containing 1 nmol of pyridoxal 5'-phosphate (PLP), 200 ng of the purified enzyme, 100 nmol of $\gamma$-cysteine, 100 nmol of dithiothreitol, and 1 μmol of O-succinyl-$\gamma$-homoserine or O-acetyl-$\gamma$-homoserine. After a 10-min incubation at 37°C, the reaction was terminated by adding 50 μl of 20% trichloroacetic acid (TCA). An aliquot of the TCA supernatant (50 μl) was added to 100 μl concentrated acetic acid and 200 μl ninhydrin solution (250 mg dissolved in 10 ml concentrated acetic acid:concentrated HCl, 60:40 [v/v]). The mixture was boiled for 10 min, and then cooled rapidly before adding 650 μl 95% ethanol (v/v). The amount of $\gamma$-cysteine was determined by measuring the optical density at 335 nm.

The reaction mixture for detecting O-acetyl-$\gamma$-homoserine sulphydrylase activity contained 100 nmol of hydrogen sulfide instead of $\gamma$-cysteine. Hydrogen sulfide was produced by the degradation of sodium hydrosulfide $n$-hydrate [17]. The detection of homocysteine was performed by high-performance liquid chromatography (HPLC) (see below). The $\beta$- or $\gamma$-lyase activity of the purified enzyme in degrading $\gamma$-cysteine, $\gamma$-cystathionine, O-succinyl-$\gamma$-homoserine, or O-acetyl-$\gamma$-homoserine was determined as previously described [17]. Briefly, the reaction mixture was centrifuged, and 250 μl of the supernatant was added to 750 μl of 0.33 M sodium acetate (pH 5.2) containing 0.017% 3-methyl-2-benzothiazoline 7-hydrazone. The reaction
mixture was then incubated at 50°C for 30 min. The amount of pyruvate and α-ketobutyrate, which were by-products of the reactions, was determined by measuring the optical density at 335 nm.

2.7. Determination of cystathionine and homocysteine

Cystathionine and homocysteine produced by enzymatic reaction were detected on a reversed-phase column (TSKgel ODS-80Ts; Tosoh) using HPLC. The reaction mixture was incubated for 2 h at 37°C. The reaction mixture was loaded onto an Amicon Microcon filter (30-kDa cutoff) to remove the enzyme, and the products were separated by ultrafiltration. The ultrafiltration product was determined after derivatization with dansylchloride as described by Tapuhi et al. [24]. The dansylated products were separated at a flow rate of 1 ml min⁻¹ with a mobile phase of 70/30 (v/v) methanol/water containing 0.6% glacial acetic acid (v/v) and 0.008% triethylamine (v/v). Excitation and emission wavelengths of 350 and 530 nm, respectively, were used to detect dansylated products.

2.8. Nucleotide sequence accession number

The sequence reported here was submitted to the EMBL and GenBank databases through the DDBJ and assigned accession number AB096931.

3. Results and discussion

The DNA region located upstream from the lcd gene encoding ßC-S lyase in S. anginosus [17] was isolated by inverse PCR, and its DNA sequence was determined. A computer-assisted search for protein-coding regions revealed two ORFs (Fig. 2A). ORF1 was a truncated gene, while the other contained the entire coding region (1092 bp long) for a 364 amino acid protein with a predicted molecular mass of approximately 40 kDa. The stop codon of this gene, named cgs, was located 2 bp upstream from the lcd gene, while the former gene was separated from the truncated ORF (ORF1) by a much greater distance (220 bp) (Fig. 2A). Inverted repeat structures forming hairpin loops, defined as $\Delta G < -6.50$ kJ mol⁻¹, were observed in the intergenic region between ORF1 and the putative cgs and in the region downstream from lcd (Fig. 2A).

A search for protein homology revealed the following sequence identities with the deduced Cgs protein: 29% with cystathionine ß-lyase (MetC) of Lactococcus lactis.
24% with cystathionine γ-synthase (MetB) of *E. coli* [26], and 13.5% with 0-acetylhomoserine sulfhydrylase (MetY) of *Le. meyeri* [6] (Fig. 3). In addition, the amino acid sequence was 16% identical to *S. anginosus* Lcd. These enzymes, associated with methionine biosynthesis (Fig. 1), belong to a family of PLP-dependent enzymes that utilize PLP as a cofactor [27]. The proposed lysine residue of the PLP binding site [28] was conserved in each
sequence (Fig. 3). It is very interesting that the deduced Cgs amino acid sequence showed significant similarities to those of proteins that have distinct functions. By contrast, the amino acid sequence of ORF1 showed a distinct number of 64% identity to that of the putative polysaccharide biosynthesis protein in *Streptococcus pneumoniae* (accession number A95178).

RT-PCR analysis was used to identify transcripts of ORF1, cgs, and *lcd* in *S. anginosus*. Two pairs of oligonucleotide primers spanning the borders of ORF1/cgs (RT-F2 and RT-R2) and cgs/lcd (RT-F4 and RT-R4) were designed (Fig. 2A). In addition, three pairs of primers were designed to amplify ORF1 (RT-F1 and RT-R1), cgs (RT-F3 and RT-R3), and *lcd* (RT-F5 and RT-R5).

The PCR product was amplified using a primer set spanning cgs/lcd. DNA fragments within ORF1, cgs, and *lcd* were also amplified. The lengths of these four products agreed with the expected sizes (939 bp with RT-F1 and RT-R1, 1095 bp with RT-F3 and RT-R3, 810 bp with RT-F and RT-R4, and 1167 bp with RT-F5 and RT-R5) (Fig. 2B). By contrast, the PCR product corresponding to the region spanning the border of ORF1/cgs was not detected. No products were observed in PCR from total RNA preparations that were not reverse-transcribed beforehand, indicating that the RT-PCR products were not derived from contaminated chromosomal DNA (Fig. 2B).

These results demonstrate that the *cgs* and *lcd* genes are cotranscribed as an operon in *S. anginosus*, while ORF1 is transcribed separately. These findings seem reasonable from the viewpoint of the homocysteine biosynthesis pathways (Fig. 1).

To evaluate the enzymatic activity of the protein encoded by the *cgs* gene from *S. anginosus*, the 1092-bp DNA ORF was cloned in-frame with GST into the pGEX-6P-1 vector. The resulting pCBL100 was used to transform competent *E. coli* BL21 cells. A single protein band was observed in SDS-PAGE analysis of the recombinant enzyme (Fig. 2C). The 40-kDa molecular mass of the denatured polypeptide agreed well with the predicted molecular mass.
We examined the function of the purified enzyme as a cystathionine $\gamma$-synthase. We developed a non-radioactive assay for cystathionine $\gamma$-synthase based on dansylated derivatization of l-cystathionine or l-homocysteine and fluorescence detection using HPLC. HPLC analysis showed that the enzyme synthesized cystathionine from l-cysteine and O-acetyl-l-homoserine (Fig. 4A). The reaction was also observed using O-succinyl-l-homoserine instead of O-acetyl-l-homoserine. An enzymatic assay showed that the relative production of l-cystathionine from l-cysteine and O-succinyl-l-homoserine was 64.5% of that of cystathionine from l-cysteine and O-acetyl-l-homoserine under the conditions described in Section 2. These results demonstrate that Cgs has cystathionine $\gamma$-synthase activity. Since S. anginosus Lcd degrades cystathionine synthesized by Cgs to form homocysteine [17], this organism clearly has the capacity to utilize the transsulfuration pathway for homocysteine biosynthesis.

Cystathionine $\gamma$-synthases from E. coli and Salmonella typhimurium have acylhomoserine sulfhydrylase activity, which produces homocysteine from O-succinyl-l-homoserine and hydrogen sulfide [29,30]. This reaction is the direct sulfhydrylation pathway for homocysteine biosynthesis (Fig. 1). HPLC analysis showed that Cgs has O-acetyl-l-homoserine sulfhydrylase activity (Fig. 4B), i.e. this enzyme could also be involved in the direct sulfhydrylation pathway. This reaction was also observed when O-succinyl-l-homoserine was used instead of O-acetyl-l-homoserine. We recently reported that the lcd product from S. anginosus has $\beta$C-S lyase activity and produces hydrogen sulfide and homocysteine from l-cysteine and l-cystathionine, respectively [17]. The lcd product from S. anginosus, which is the only enzyme responsible for the formation of hydrogen sulfide in this organism [17], has an extremely high capacity to produce hydrogen sulfide, compared with enzymes from the other streptococci [18]. This activity of Lcd, together with the O-acetyl-l-homoserine sulfhydrylase activity of Cgs, suggests that S. anginosus utilizes the direct sulfhydrylation pathway for homocysteine biosynthesis, as well as the transsulfuration pathway. However, the capacity to utilize both pathways is not an advantage for S. anginosus because both Cgs and Lcd are necessary for the formation of homocysteine, if either the transsulfuration pathway or direct sulfhydrylation pathway is utilized.

In addition to being able to synthesize cystathionine and homocysteine, S. typhimurium cystathionine $\gamma$-synthase also degrades l-cystathionine, O-succinyl-l-homoserine, and O-acetyl-l-homoserine via a $\beta$- or $\gamma$-elimination reaction [31]. We examined the function of the Cgs as a cystathionine $\beta$- or $\gamma$-lyase. Cystathionine $\beta$-lyase catalyzes the conversion of cystathionine to ammonia, pyruvate, and homocysteine [32]. This enzyme also has the capacity to cleave sulfur amino acids containing a $\beta$C-S linkage, such as l-cysteine, producing pyruvate, ammonia, and sulfur-containing molecules [33]. By contrast, cystathionine

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**Fig. 4.** Reversed-phase HPLC profiles of dansylated reaction products. A: Demonstration of cystathionine $\gamma$-synthase activity catalyzed by Cgs. Reaction mixtures contained O-acetyl-l-homoserine and l-cysteine in the absence (top) or presence (bottom) of Cgs. B: Demonstration of O-acetyl-l-homoserine sulfhydrylase activity catalyzed by Cgs. Reaction mixtures contained O-acetyl-l-homoserine and hydrogen sulfide in the absence (top) or presence (bottom) of Cgs. Arrowheads indicate the elution times of cysteine, cystathionine, and homocysteine.
γ-lyase converts cystathionine to cysteine, α-ketobutyrate, and ammonia. Based on this information, the abilities of Cgs to degrade L-cystathionine and L-cysteine were tested by assaying the generation of pyruvate or α-ketobutyrate [34]. Neither pyruvate nor α-ketobutyrate was detected under the experimental conditions used. By contrast, in S. anginosus [34]. Neither pyruvate nor α-ketobutyrate was detected under the experimental conditions used. By contrast, incubation of O-succinyl-L-homoserine or O-acetyl-L-homoserine with the Cgs resulted in the formation of α-ketobutyrate. These findings suggest that cystathionine γ-synthase from S. anginosus acts as a γ-lyase. Unlike S. typhimurium cystathionine γ-synthase, however, S. anginosus cystathionine γ-synthase has no capacity to degrade cystathionine. In S. anginosus, the protein encoded by lcd (βC-S lyase) has this capacity instead of cystathionine γ-synthase [17].

Therefore, the function of S. anginosus Cgs is similar to that of E. coli and S. typhimurium MetBs. The amino acid sequence of E. coli metB showed 96% identity to that encoded by the metB homolog in S. typhimurium [35]. However, the amino acid sequence of S. anginosus Cgs did not show extremely high similarity to that of E. coli MetB, compared with those of L. lactis MetC and Le. meyeri MetY (Fig. 3). Moreover, there are no common sequences identified only in S. anginosus Cgs and E. coli MetB. It is very interesting that the overall amino acid sequences of enzymes with different functions show considerable similarity. This suggests that these different enzymes might have similar origins or histories. By contrast, S. anginosus Lcd showed no significant identity to these four proteins (less than 4%), although its function is quite similar to that of MetC. It seems reasonable to postulate that the functions of PLP-dependent enzymes cannot be predicted from their amino acid sequences.

Although sulfur amino acids can be a source of hydrogen sulfide and methyl mercaptan, which are highly toxic to mammalian cells [36] and are the main causes of oral malodor [37], little attention has been paid to information regarding the biosynthetic pathways of sulfur amino acids in oral bacteria, such as streptococci. Further studies are necessary to clarify the mechanisms of sulfur amino acid production and the relationship between the amino acid sequences of PLP-dependent enzymes and their function.

Acknowledgements

This study was supported in part by a grant from the Takeda Science Foundation (Y.N.) and Grants-in-Aid for the Encouragement of Young Scientists 13771265 (Y.N.) and 14771185 (Y.Y.) from the Ministry of Education, Culture, Sports, Science, and Technology, of Japan.

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