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Determination of a functional lysine residue of a plant cysteine synthase by site-directed mutagenesis, and the molecular evolutionary implications

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Comparison of seven deduced amino acid sequences of cysteine synthase (*O*-acetyl-L-serine (thiol)-lyase, EC 4.2.99.8) from plants and bacteria disclosed the presence of 12 conserved Lys residues, which can be candidates for a functional binding site for pyridoxal phosphate cofactor. These 12 conserved Lys residues in a cDNA clone encoding spinach cysteine synthase A were replaced with Gly by oligonucleotide-directed in vitro mutagenesis. These Lys → Gly mutated cDNAs were transferred into *Escherichia coli* NK3, a cysteine auxotroph lacking both cysteine synthase loci, *cysK* and *cysM*. One mutant replaced at Lys-49 could not complement the cysteine requirement of NK3, whereas other mutants and wild-type clone could. No enzymatic activity of cysteine synthase A was detected either in the cell-free extracts of *E. coli* NK3 transformed with the Lys-49 mutant. These results indicated that Lys-49 is a functional residue for the catalytic activity of cysteine synthase. This Lys residue is conserved in other evolutionarily related amino acid-metabolizing enzymes catalyzing reactions involving the β-carbon of amino acids.

Cysteine synthase; Genetic complementation; Pyridoxal phosphate; *Spinacia oleracea*

1. INTRODUCTION

Cysteine synthase (CSase; *O*-acetyl-L-serine (thiol)-lyase, EC 4.2.99.8) catalyzes the formation of L-cysteine from *O*-acetyl-L-serine and hydrogen sulfide concomitant with the release of acetic acid [1]. This enzyme requires one molecule of pyridoxal phosphate (PLP) per subunit of apo-protein of around M_r 35 kDa [2]. The reaction proceeds with the formation of an aldimine intermediate of *O*-acetyl-L-serine and PLP which is bound to the enzyme protein through the amino group of a functional Lys by Schiff base formation.

Multiple forms of the CSase family are present in plant cells [3,4]. Several cDNA clones for these different forms of CSase have recently been isolated by us [5,6] and other groups [7–9]. The predicted amino acid sequences of these cDNAs show ~ 70% homology with each other in their coding regions. It would be interesting to determine a functional Lys site for the catalytic activity of CSase.

In the present study, we have identified an active Lys residue for catalytic action of CSase A from spinach (*Spinacia oleracea* L.) by site-directed mutagenesis of the cDNA clone. We found that this Lys residue is highly conserved in the group of amino acid-metabolizing enzymes catalyzing the modification of the β-carbon of amino acids.

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Abbreviations. CSase, cysteine synthase; PLP, pyridoxal phosphate.

2. MATERIALS AND METHODS

2.1. *Plasmids and bacteria*

The expression plasmid, pKM1 [5], contained a full-length cDNA of spinach CSase A under the *lacZ* promoter of pTV118N (Takara, Kyoto). *Escherichia coli* NK3 (*ΔtrpE5 leu-6 thi hsdR hsdM⁺ cysK cysM*), a cysteine auxotroph, lacked two CSase loci (*cysK* and *cysM*) and was used for transformation and complementation.

2.2. *Site-directed mutagenesis*

The 25-mer oligonucleotides shown in Table I were synthesized for the mutagenesis of 12 conserved Lys residues into Gly. The sequences of synthetic oligonucleotides were predicted to have homology only with the target sequences and not with other sequences in the cDNA clone. Oligonucleotide-directed mutagenesis was carried out with single-stranded DNA of pKM1 by the Eckstein method using a kit supplied by Amersham (Bucks, UK). These point mutations were verified by dideoxy sequencing for a ca. 250 bp region around the mutagenized site.

2.3. *Assay of enzymatic activity and Western blot analysis*

E. coli NK3 transformed with the mutant plasmids was cultured in LB medium [10] for 2 h; isopropyl β-D-thiogalactoside was added to 1 mM, and the incubation was continued for 3 h. Soluble protein extracts were obtained and assayed for CSase activity as described previously [5]. Western blot analysis of the protein extracts was also performed as reported [5].

2.4. *Genetic complementation of E. coli RM NK3 by mutant plasmids*

Transformed *E. coli* NK3 with the mutant plasmids was cultured on a minimal M9 agar plate [10] supplemented with 0.1 mM isopropyl β-D-thiogalactoside, 0.02% leucine and tryptophan. The plates were incubated at 37°C for 5 days for the assay of cysteine requirement for bacterial growth.

3. RESULTS AND DISCUSSION

Comparison of seven deduced amino acid sequences

Table I

Synthetic oligonucleotides for site-directed mutagenesis to replace 12 conserved Lys with Gly

No	Name and position[a]	Sequence[b]
1	K38G	5′-GTGTTGCTGCAGGGCTGGAAGGAAT-3′
2	K49G	5′-GCTCTAGTGTTGGAGACAGGATTGG-3′
3	K62G	5′-CTGATGCTGAAGGAAGCGGGCTTAT-3′
4	K95G	5′ CTAAAGGTTACGGGCTCATCATTAC-3′
5	K126G	5′-CTGATCCAGCAGGAGGTATGAAAGG-3′
6	K129G	5′-CAAAAGGTATGGGAGGGGCTGTTCA-3′
7	K134G	5′-GGGCTGTTCAGGGGGCTGAGGAGAT-3′
8	K197G	5′-GAAAATACCTAGGGGAACAAAACCC-3′
9	K220G	5′-TGTCTGGAGGAGGACCTGGCCCACA-3′
10	K225G	5′-CTGGCCCACATGGGATTCAAGGACT-3′
11	K282G	5′-CCGCTGCCATTGGAGTGGCAAAGAG-3′
12	K292G	5′-AAAATGCTGGAGGACTCATCGTCGC-3′

[a] Number represents the position of the Lys residue replaced.
[b] Underline indicates the position of nucleotides mutagenized

revealed 12 conserved Lys residues in the CSases from spinach [5,6], bell pepper (*Capsicum annuum*) [7], wheat (*Triticum aestivum*) [8], *cysK* of *E. coli* and *Salmonella typhimurium* [11,12], and *cysM* of *E. coli* [13] (Fig. 1). The codons for these 12 Lys residues were replaced with the codons for Gly by oligonucleotide-directed mutagenesis in the cDNA of pKM1.

E. coli NK3, a cysteine auxotroph lacking the endogenous CSase loci *cysK* and *cysM*, was transformed with these plasmids containing the mutated CSases. Western blot analysis for the protein extracts of transformed *E. coli* NK3 using anti-CSase A antibody [5] indicated that nearly the same amount of immunoreactive CSase proteins accumulated in NK3 cells (data not shown).

The enzymatic activity of CSases was determined in

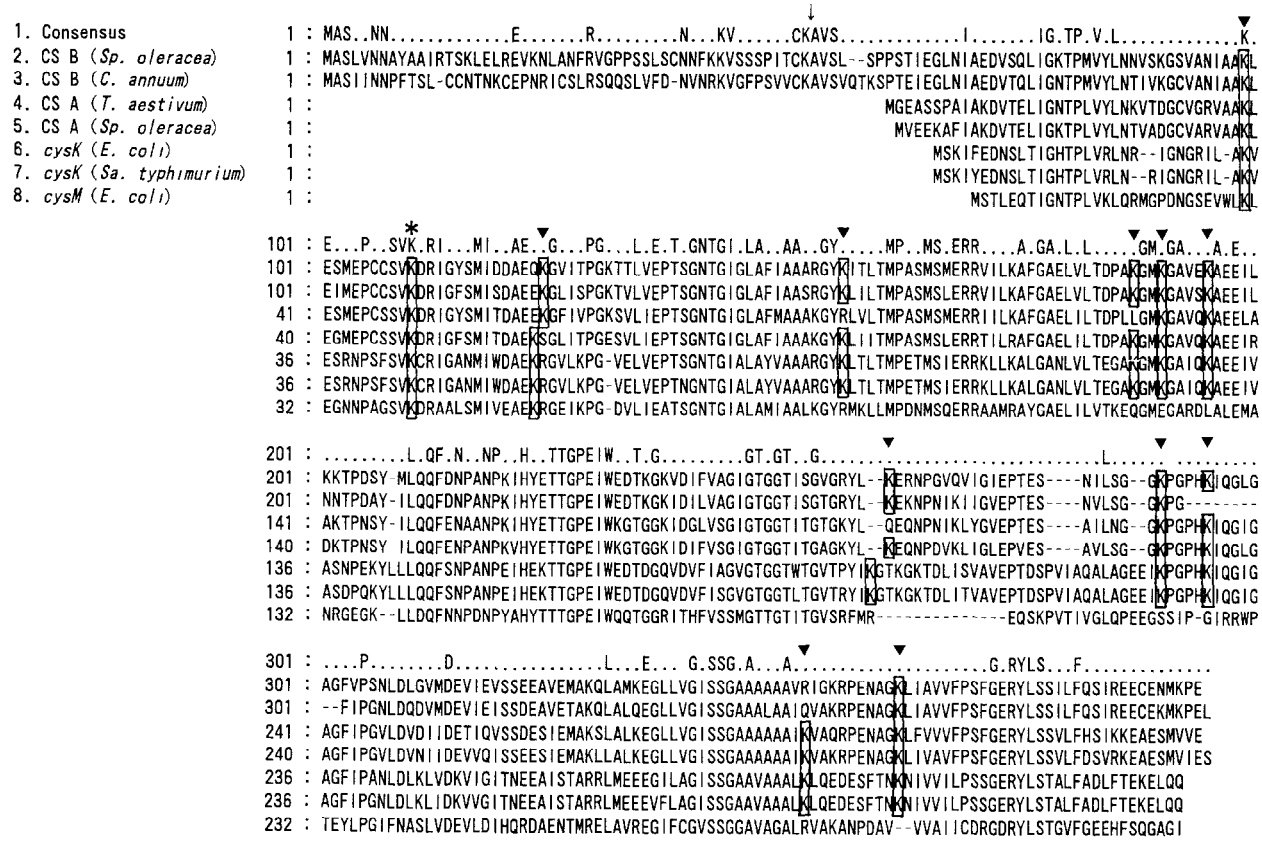

Fig. 1. Comparison of consensus and deduced amino acid sequences of CSases from various sources. Twelve conserved Lys residues (▼) are indicated by boxes and were mutagenized with synthetic oligonucleotides. The asterisk indicates the position of functional Lys-49. The arrow indicates the cleavage site of transit peptide of CSase B [6]. Dashes indicate gaps in the sequence for the best alignment

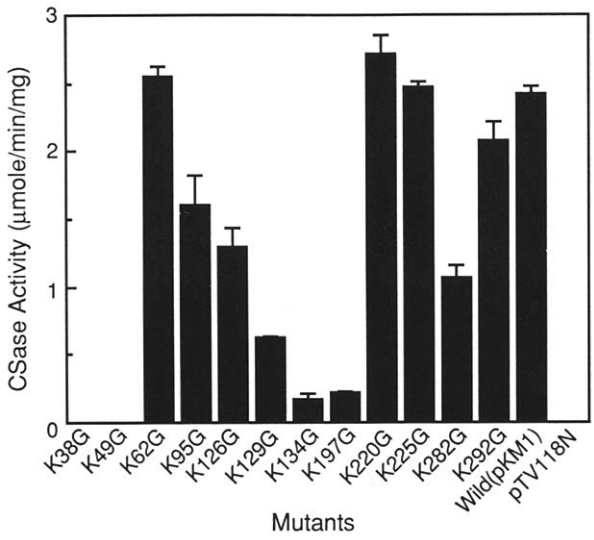

Fig 2. CSase activity in extracts of *E. coli* NK3 transformed with mutant plasmids. The soluble protein extracts of NK3 cells transformed with 12 plasmids containing the site-directed mutation, pKM1 harboring the wild cDNA as a positive control, and pTV118N without the cDNA as a negative control, were subjected to enzyme assay. Bars show S.D. ($n = 3$).

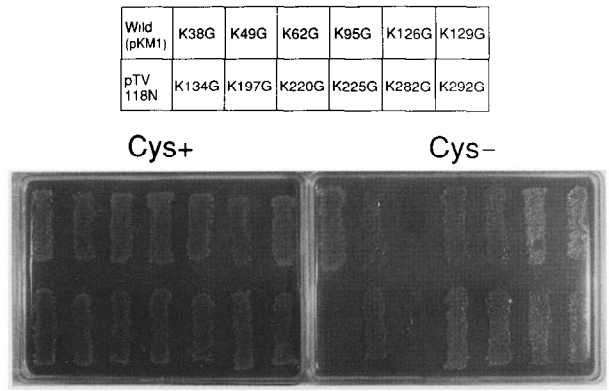

Fig. 3. Assay for genetic complementation of Cys- *E coli* NK3 by transformation with expression vectors carrying mutated CSase cDNA. The growth of transformed bacteria was assayed on a M9 minimal agar plate supplemented with 0.02% leucine and tryptophan plus 0.5 mM cysteine (left plate) or without cysteine (right plate). pKM1 harboring the wild-type cDNA and pTV118N were used as a positive and a negative control, respectively.

the protein extracts of the transformed NK3 cells (Fig. 2). No detectable CSase activities were observed in the mutants K38G and K49G. The other 10 mutants had the CSase activities by in vitro assay, although the activities were in the range from 7% to 115% of that of wild-type CSase in pKM1.

The ability of genetic complementation in an auxotrophic *E. coli* NK3 was examined for these mutant plasmids (Fig. 3). Only the mutant K49G could not complement the cysteine requirement of the NK3 cells, but other mutants, including K38G, could. These results clearly indicated that Lys-49 is a functional residue responsible for CSase activity.

The Lys-49 in spinach CSase A corresponds to Lys-45 in *S. typhimurium cysK* product in Fig. 1. This residue was also shown to be the PLP-binding site by peptide sequencing of the proteolytically digested fragment of the *S. typhimurium* CSase [14]. This previous investigation also supported our conclusion that this residue is responsible for the enzyme activity as the PLP-binding site. As shown in Fig. 1, the Lys-49 is highly conserved among all 7 CSases for which the deduced amino acid sequences are available.

Interestingly, the region around Lys-49 is also conserved in evolutionarily related enzymes for amino acid metabolism [15,16] and in rat hemoprotein H-450 [17] (Fig. 4). The reactions catalyzed by these amino acid-metabolizing enzymes involve a common aminoacrylate form as a key reaction intermediate. Stereochemical courses of the reactions concerning toheβ-carbon of amino acids are also the same in the reactions catalyzed by these enzymes, as far as experimentally examined [18]. These results suggested that these PLP-dependent enzymes have evolved from a common ancestor enzyme conserving this particular Lys residue for the PLP binding center and the reaction mechanism. Hemoprotein H-450 from rat liver cytosol is reported to have no enzymatic activity of amino acid metabolism [17]. However, this particular Lys residue is conserved, suggesting the presence of specific role of this residue for an unknown function of the H-450 protein.

The residue corresponding to Lys-38 in spinach CSase A is also highly conserved. It is assumed that these two Lys residues (Lys-49 and Lys-38) are impor-

			Reference
Consensus		...K.E...P..GS.K.R..	
Cysteine synthase (Isoform A, *S. oleracea*)	(35)	VAAKLEGMEP.CSSVKDRIG	[5]
Hemoprotein H-450 (Rat)	(102)	LLAKCEFFNA.GGSVKDRIS	[17]
Threonine synthase (*E. coli*)	(93)	VGCLELFHGP.TGSFKDFGG	[15]
Threonine dehydratase (*Saccharomyces cerevisiae*)	(95)	VILKREDLLP.VFSFKIRGA	[15]
Serine dehydratase (*E. coli*)	(103)	LLLKKDSHLPISGSIKARGG	[15]
Tryptophan synthase (*E. coli*)	(72)	LYLKREDLLH.GGAHKTNQV	[16]
		↑	

Fig. 4. Comparison of a conserved region in the amino acid sequences of related amino acid-metabolizing enzymes and of hemoprotein H-450 around the PLP-binding Lys residue. Numbers in parentheses indicate the location of each first amino acid of the region in the respective complete sequences. Consensus indicates matching amino acids in at least four sequences among the six proteins.

tant for the catalytic action of PLP-dependent enzymes and consequently have been conserved in evolution. For catalytic action of PLP-dependent enzymes, another basic residue beside the PLP-binding site is assumed to be necessary to form a salt bridge with the phosphate group of PLP. The ε-amino group of Lys-38 is likely to play the role of the ionic counterpart of the phosphate group of PLP. The mutants, K134G and K197G, showed weak enzymatic activity of CSase and reduced complementation ability. Tertiary structures of these mutated proteins might have been changed to cause reduced catalytic activity of CSase. Further investigation is necessary to clarify the three-dimensional structure of the CSase protein.

In conclusion, we determined a highly conserved Lys residue in a plant CSase as a functional amino acid residue by a genetic method involving the site-directed mutagenesis technique.

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