Cystathionine γ -synthase from *Arabidopsis thaliana*: purification and biochemical characterization of the recombinant enzyme overexpressed in *Escherichia coli*

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Cystathionine γ -synthase catalyses the first reaction specific for methionine biosynthesis in plants, the γ -replacement of the phosphoryl substituent of *O*-phosphohomoserine by cysteine. A cDNA encoding cystathionine γ -synthase from *Arabidopsis thaliana* has been cloned and used to overexpress the enzyme in *Escherichia coli*. The native recombinant enzyme is a homotetramer composed of 53 kDa subunits, each being tightly associated with one molecule of pyridoxal 5'-phosphate that binds at lysine-379 of the protein precursor. The replacement reaction follows a Ping Pong mechanism with a $V_{\max}$ of 33.6 units/mg and K_m values of 2.5 mM and 460 μ M for *O*-phosphohomoserine and cysteine respectively. The protective effect of *O*-phosphohomoserine against enzyme inactivation by propargyl-

glycine indicated that the K_d for the substrate is approx. 1/2500 of its K_m value. Thus most of these biochemical properties are similar to those previously reported for plant and bacterial cystathionine γ -synthases. However, the plant enzyme differs markedly from its enterobacterial counterparts because it catalyses a very faint γ -elimination of *O*-phosphohomoserine in the absence of cysteine, this process being about 1/2700 as fast as the γ -replacement reaction and approx. 1/1500 as fast as the γ -elimination catalysed by the *E. coli* enzyme. This huge difference could be attributed to the inability of the *A. thaliana* cystathionine γ -synthase to accumulate a long-wavelength-absorbing species that is characteristic for the efficient γ -elimination reaction catalysed by the enterobacterial enzyme.

INTRODUCTION

Methionine biosynthesis in higher plants consists of three consecutive reactions catalysed by cystathionine γ -synthase (EC 4.2.9.99), cystathionine β -lyase (EC 4.4.1.8) and methionine synthase (EC 2.1.1.14) (Scheme 1) [1,2]. The first two enzymes

are part of the trans-sulphuration reactions consisting of the transfer of the sulphur atom of cysteine to homocysteine with the thioether cystathionine as intermediate. Cystathionine γ -synthase is the only known enzyme with a physiological function of catalysing a replacement reaction at the γ -carbon of an amino acid, i.e. the replacement of an electronegative group by another one on the fourth carbon of an amino acid. The overall reaction catalysed by cystathionine γ -synthase is:

Scheme 1 Synthesis of aspartate-derived amino acids in plants

Abbreviations used: DTT, dithiothreitol; IPTG, isopropyl β -D-thiogalactoside; OAH, *O*-acetylhomoserine; OPH, *O*-phosphohomoserine; OSH, *O*-succinylhomoserine; PAG, propargylglycine; PLP, pyridoxal 5'-phosphate; SAM, S-adenosylmethionine; TFA, trifluoroacetic acid.

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the γ -replacement reaction follows a Ping Pong mechanism where the homoserine ester binds first [4,7]. The physiological function of bacterial cystathionine γ -synthase is the catalysis of the γ -replacement reaction leading to cystathionine synthesis. However, the enzyme from enterobacteria efficiently decomposes OSH in the absence of cysteine by a γ -elimination reaction producing α -ketobutyrate, succinate and ammonia, the turnover rate of the γ -elimination reaction being only one-half to one-fifth that of the γ -replacement reaction [4,6].

In plants, cystathionine γ -synthase is located in the stromal space of chloroplasts [8,9]. The enzyme has been purified from spinach leaf chloroplasts and some of its biochemical properties have been examined [9]. Plant cystathionine γ -synthase is a typical PLP-containing protein that exhibits an absorption maximum at 425 nm and exists as a homotetramer of 53 kDa subunits. The plant enzyme differs markedly from its bacterial counterpart in that it uses *O*-phosphohomoserine (OPH) as the physiological substrate for cystathionine biosynthesis. This property is of particular interest because OPH is also the direct precursor for threonine synthesis (Scheme 1). Thus the common route for methionine and threonine syntheses in plants terminates with OPH, whereas homoserine is the branched point between these pathways in bacteria [1]. *In vitro*, plant cystathionine γ -synthase is able to catalyse a direct sulphydration of OPH when cysteine is replaced by sodium sulphide [9,10]. *In vivo*, this reaction leading to homocysteine synthesis with a bypass of the cystathionine intermediate is not, however, assumed, to participate in methionine synthesis because a mutant of *Nicotiana plumbaginifolia* that is defective in cystathionine β -lyase requires homocysteine or methionine for growth [11].

When we initiated this work the only molecular data on plant cystathionine γ -synthase were provided by the sequencing of an *Arabidopsis thaliana* genomic fragment containing the 3' end of a gene exhibiting significant similarity to *metB* [12]. Recently a cDNA clone and the corresponding gene coding cystathionine γ -synthase have been obtained from *A. thaliana* [13]. The sequence encodes a 60 kDa polypeptide that is 35% identical with the *E. coli metB* product and contains a putative plastid transit peptide.

There are several reasons to support a better characterization of a plant cystathionine γ -synthase: (1) cystathionine γ -synthase seems to be the key regulatory step in methionine synthesis [1]; (2) methionine seems to control the carbon flux through threonine and the resulting branched-chain amino acids via the activation of threonine synthase by *S*-adenosylmethionine (SAM) [14,15]; and (3) cystathionine γ -synthase is potentially a suitable target for new herbicides. Because the enzyme is present in trace amounts in plant tissues [9], the recent molecular characterization of cystathionine γ -synthase in *A. thaliana* [13] offers the possibility to express a plant cDNA in bacteria and to initiate a detailed analysis of the biochemical properties of the plant enzyme. In the present study we describe the cloning and the overexpression in *E. coli* of an *A. thaliana* cDNA encoding a functional cystathionine γ -synthase. We also report a purification procedure for the isolation of the recombinant enzyme and analyse its biochemical properties in detail.

MATERIALS AND METHODS

Materials

OPH and OAH were synthesized and purified as described previously [9]. NADH and lactate dehydrogenase were obtained from Boehringer-Mannheim (France). Synthetic oligonucleotides, dithiothreitol (DTT) and isopropyl β -D-thiogalactoside

(IPTG) were from Bioprobe Systems. All other biochemicals were obtained from Sigma Chimie SARL (La Verpillère, France).

Plant material and bacterial strains

A. thaliana (var. *columbia*) plants used for the preparation of RNA and protein extracts were grown in soil for 3 weeks under a 12 h photoperiod of white light from fluorescent tubes (10–40 μ E/m²) at 25 °C.

DNA manipulations were conducted in *E. coli* DH10B cells (Gibco BRL) and expression of recombinant proteins was performed in the *E. coli* strain BL21(DE3)pLysS (Novagen).

cDNA cloning

Total RNA (1 μ g) from whole *A. thaliana* plants was isolated by the guanidinium thiocyanate procedure [16] and reversed transcribed with Moloney murine leukaemia virus reverse transcriptase at 42 °C for 90 min. Second-strand cDNA synthesis and adaptor ligation were performed with the Marathon[®] cDNA amplification kit (Clontech). The cDNA library was amplified by 25 cycles of PCR with the reverse primer RCGS3' (5'-CAATAGTTCAAAGACATCATTATTACGG-3'), which is complementary to the 3' end of the *AtcysI* gene [12], and forward primers corresponding to the adaptors (Clontech). Amplified products were cloned in pZErO[®]-1 (Invitrogen), digested by *EcoRV* and sequenced on both strands by automatic fluorescence-based sequencing (Genome Express, Grenoble, France).

Construction of fusion and mature cystathionine γ -synthase expression plasmids

The pMAL CGS construct coding for a 96 kDa fusion of cystathionine γ -synthase and maltose-binding protein was obtained through PCR mutagenesis with pZErO ATCGS as a template. Primers pMALCGS5' (5'-GAGATCTAGATTTTCCT-CCTAATTTTCG-3') and pMALCGS3' (5'-GAGAGAAGCTT-AAAGGACAAAGAAACGAC-3') were used to amplify the coding sequence of the *A. thaliana* cystathionine γ -synthase devoid of its 62 N-terminal residues. The amplification product was digested by *XbaI* and *HindIII* and subcloned into the pMAL-p2 vector (BioLabs) digested by the same enzymes.

With pZErO ATCGS as a template, PCR amplification was performed with the primers pMALCGS3' and pETCGS5' (5'-GAGACATATGGTCCGTCCAGTCAGC-3'), which introduced a *NdeI* restriction site containing an ATG translation-initiation codon directly before the codon corresponding to the first residue of the mature protein (Val-69). The amplification product was digested by *NdeI* and *HindIII* for further cloning into the pET29a(+) vector (Novagen) digested by the same enzymes. The DNA insert was further sequenced to ensure that no mutation had been introduced during the course of the PCR amplification.

Expression and purification of recombinant cystathionine γ -synthases

The *E. coli* BL21(DE3)pLysS strain transformed with pMAL CGS was grown at 37 °C in Luria broth supplemented with carbenicillin (100 μ g/ml) and chloramphenicol (25 μ g/ml). When D_{600} reached 0.6, 1 mM IPTG was added and growth continued at 28 °C for 3 h. Cells were collected by centrifugation (2000 g for 15 min), resuspended in 20 mM Tris/HCl

(pH 7.5)/1 mM DTT/1 mM EDTA/5 mM ϵ -aminocaproic acid/1 mM benzamidine/1 mM PMSF and disrupted by sonication for 10 min at 0 °C. After a 15 min centrifugation at 40000 *g* to remove cell debris, the soluble extract was supplemented with 0.5 M NaCl and 0.05 % (v/v) Tween 20, and applied to an amylose column (2.6 cm $\times$ 12 cm; BioLabs). The fusion protein was specifically eluted from the column by using the loading buffer supplemented with 10 mM maltose and cleaved with factor Xa (BioLabs) in accordance with the manufacturer's instructions. Cystathionine γ -synthase was resolved from the maltose-binding protein by anion-exchange chromatography on a MonoQ HR 5/5 column (Pharmacia) and used for rabbit immunization (Centre Valbex, Villeurbanne, France) with three injections each of 200 μ g of enzyme every 3 weeks.

E. coli BL21 (DE3)pLysS cells transformed with pET MCGS were grown and induced as described above in Luria broth supplemented with kanamycin (50 μ g/ml), chloramphenicol (25 μ g/ml), pyridoxine (10 μ M) and thiamin (15 μ M); 3 h after induction, cells were harvested by centrifugation and resuspended in buffer A containing 50 mM Tricine/NaOH, pH 7.5, 1 mM EDTA, 5 mM ϵ -aminocaproic acid and 1 mM benzamidine. Cells were disrupted by sonication in the presence of 1 mM PMSF and centrifuged at 40000 *g* and 4 °C for 15 min to yield the soluble extract (950 mg of protein starting from 3 litres of cell culture). Nucleic acids were precipitated by the addition of 0.2 % streptomycin sulphate and the supernatant was supplemented with crystalline $(\text{NH}_4)_2\text{SO}_4$ to 50 % satn. The resulting precipitate was collected by centrifugation (40000 *g* for 15 min at 4 °C) and resuspended in buffer A supplemented with 2.5 M NaCl. The protein extract (485 mg) was clarified by centrifugation and applied to a phenyl-Sepharose CL4B column (2.6 cm $\times$ 12 cm; Pharmacia) equilibrated in buffer A containing 2.5 M NaCl. After being loaded, the column was washed in the equilibration buffer and proteins were eluted with a 500 ml linear gradient from 2.5 to 0 M NaCl in buffer A (2 ml/min, 5 ml fractions). Fractions containing the highest activity were pooled and supplemented with $(\text{NH}_4)_2\text{SO}_4$ to yield an 80 %-satd. solution. The solution was centrifuged at 40000 *g* and 4 °C for 15 min and the resulting pellet was resuspended in buffer A. The extract (110 mg of protein) was desalted by passage through a PD10 Sephadex G25 (M) column (Pharmacia) and applied to a Source Q column (1 cm $\times$ 10 cm, Pharmacia) equilibrated in buffer A. After the column had been washed with buffer A, proteins were eluted with a 180 ml linear gradient from 0 to 0.35 M NaCl in buffer A (0.75 ml/min, 1.5 ml fractions). The active fractions were pooled (11 mg of protein), concentrated to 1.5 ml with Macrosep-10 tubes (Filtron) and injected on a Superdex 200 column (1.6 cm $\times$ 60 cm, Pharmacia) equilibrated in buffer A containing 0.15 M NaCl. Elution was conducted in the equilibration buffer at 1 ml/min, and 1.5 ml fractions were collected.

Immunopurification of cystathionine γ -synthase from *A. thaliana* leaves

A. thaliana leaves (300 g) were homogenized in 500 ml of buffer B [50 mM Tricine/NaOH (pH 7.5)/1 mM DTT/1 mM EDTA/50 μ M PLP] supplemented with a mixture of protease inhibitors (5 mM ϵ -aminocaproic acid/1 mM benzamidine/1 mM PMSF) with a Waring blender operating at full speed. The homogenate was filtered through muslin, passed through a 20 μ m nylon mesh and centrifuged at 28000 *g* for 30 min to remove cell debris and membranes. Crystalline $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant until 80 % satn. was achieved; the solution was stirred for 30 min at 4 °C. Proteins were collected by centrifugation (28000 *g* for

30 min), resuspended in extraction buffer, clarified by centrifugation (40000 *g* for 15 min) and desalted by passage through a Sephadex G25 (M) column (2.6 cm $\times$ 30 cm; Pharmacia) previously equilibrated in buffer B containing 1 mM benzamidine and 5 mM ϵ -aminocaproic acid. This crude protein extract (2 g of protein; 30 ml) was applied to a Fractogel EMD DEAE 650 (M) column (2.6 cm $\times$ 30 cm; Merck) equilibrated in buffer B. Elution of cystathionine γ -synthase activity was performed with a 700 ml linear gradient from 0 to 0.3 M NaCl in buffer B (flow rate 1.5 ml/min; fraction size 6 ml). Fractions containing the highest activity were combined and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (80 % satn.). Proteins (180 mg) were collected by centrifugation (48000 *g* for 20 min), resuspended in buffer C [20 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 mM EDTA/0.5 % Triton X-100/5 mM ϵ -aminocaproic acid/1 mM benzamidine] and passed through a Sephadex G25 (M) column (1.6 cm $\times$ 25 cm; Pharmacia) equilibrated in the same buffer.

Anti-(cystathionine γ -synthase) IgGs were prepared from the immune serum by $(\text{NH}_4)_2\text{SO}_4$ fractionation and anion-exchange chromatography on a DEAE Trisacryl column (Sepracor) and then covalently attached to Protein A-agarose beads [17]. Beads containing approx. 1.5 mg of IgG were incubated with the above protein fraction for 16 h at 4 °C, washed in buffer C and centrifuged at 14000 *g* for 5 min. The pellet was boiled in SDS sample buffer, resolved by SDS/PAGE and blotted to a Pro-Blot sequencing membrane (Applied Biosystems) [18]. Polypeptides were revealed by staining with Coomassie Blue R-250, cut out from the membrane and subjected to Edman degradation with a Model 494 gas-liquid-phase protein sequencer (Applied Biosystems).

Enzyme assays

Cystathionine γ -synthase activity was assayed as described by Ravanel et al. [9] with the Malachite Green method as a measure of released phosphate, or the *O*-phthaldialdehyde derivatization procedure as a measure of cystathionine production. The assay mixture (100 μ l) contained 20 mM Mops/NaOH, pH 7.5, 1 mM DTT, 1 mM cysteine and 10 mM OPH. Reactions were conducted at 30 °C for 1–5 min in the presence of 0.04–0.2 μ g of purified enzyme (7.5–37.7 nM on a subunit basis). One unit of activity corresponded to the amount of enzyme that catalysed the formation of 1 μ mol of product/min.

The ability of recombinant cystathionine γ -synthase to catalyse the γ -elimination of homoserine derivatives was assayed by determining α -ketobutyrate in the presence of lactate dehydrogenase and NADH [4]. The reaction mixture (100 μ l) contained 20 mM Mops/NaOH, pH 7.5, 10 mM homoserine ester and 5–25 μ g of purified cystathionine γ -synthase (0.94–4.7 μ M on a subunit basis). The reaction developed at 30 °C and aliquots were withdrawn at time intervals and transferred to a reaction cuvette containing 50 mM Tricine/NaOH, pH 7.5, 200 μ M NADH and 70 units of lactate dehydrogenase. Reactions were conducted at 30 °C and α -ketobutyrate formation was monitored at 340 nm through NADH oxidation.

Inhibitor studies

Inactivation experiments of recombinant cystathionine γ -synthase by D,L-propargylglycine (PAG) were conducted at 25 °C as follows. The inactivation reaction was initiated by the addition of purified cystathionine γ -synthase (2 μ g, 0.19 μ M subunit) to a 200 μ l solution containing various concentrations of the inhibitor (0–200 μ M) in 20 mM Mops/NaOH, pH 7.5. At intervals, 20 μ l aliquots were removed from the preincubation mixture and

assayed for residual enzyme activity. Data were fitted to the appropriate theoretical equations by using the KaleidaGraph® program (Abelbeck software).

Spectroscopic determinations

Steady-state absorption spectra for the reaction of cystathionine γ -synthase with substrates or inhibitor were recorded at 30 °C in quartz cuvettes with an 1 cm optical path length, on a UVikon 860 (Kontron) spectrophotometer. Initial absorption spectra were usually recorded with approx. 20 μ M (subunit) cystathionine γ -synthase in 50 mM Tricine/NaOH, pH 7.5, and additions never exceeded 2 % of the assay volume.

Purification of the PLP-binding peptide

Purified recombinant cystathionine γ -synthase (1 mg in 1 ml of 50 mM Tricine/NaOH, pH 7.5) was reduced in the presence of 0.6 % NaBH₄ for 15 min at 0 °C. The reaction was stopped by adding 10 μ l of acetic acid and the reduced protein was desalted through a PD10 Sephadex G25 (M) column equilibrated in 0.1 M Tris/HCl (pH 8.5)/2 mM DTT. Crystalline urea was added to a final concentration of 8 M to the protein solution and the mixture was incubated for 10 min at 30 °C before the addition of iodoacetamide (final concentration 20 mM). The mixture was incubated for 15 min at 30 °C and the protein was passed through a Sephadex G25 (M) column equilibrated in 0.1 M Tris/HCl, pH 8.2. The borohydride-reduced and carboxymethylated protein was incubated for 16 h at 37 °C in the presence of 40 μ g of tosylphenylalaninechloromethane-treated trypsin (Sigma). This solution was freeze-dried and peptides resulting from the cleavage were solubilized in 200 μ l of 0.1 % (v/v) trifluoroacetic acid (TFA). The pyridoxyl peptide was further purified by reverse-phase HPLC on a μ Bondapak C₁₈ column (3.9 mm $\times$ 300 mm, 10 μ m particle size, 30 nm pore; Millipore-Waters) with a Waters HPLC system connected to a Kontron SFM25 fluorimeter and a Waters 484 UV detector. Mobile phases were: solvent A, 0.1 % (v/v) TFA in distilled water and solvent B, 0.07 % (v/v) TFA in 80 % (v/v) acetonitrile. Peptides were eluted from the column with a 90 min linear gradient from 0 to 80 % solvent B at a flow rate of 0.5 ml/min. The effluent was monitored by UV absorbance (210 nm) and fluorescence (excitation 330 nm and emission 395 nm) measurements. The purified phosphopyridoxyl peptide was further freeze-dried and sequenced.

Protein determination, electrophoresis and immunoblotting

Protein was measured either by the method of Bradford [19] with Bio-Rad protein assay reagent, with bovine γ -globulin as standard, or by measuring A_{205} [20]. SDS/PAGE was performed at room temperature in slab gels containing a linear 7.5–15 % (w/v) polyacrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were as detailed by Chua [21]. For Western blot analysis, the separated polypeptides were transferred electrophoretically to nitrocellulose sheets (Bio-Rad) by the method of Towbin et al. [22] with a trans Blot Cell (semidry system; Bio-Rad). Cystathionine γ -synthase protein detection was performed with rabbit polyclonal antibodies raised against the recombinant protein and horseradish peroxidase-coupled goat anti-(rabbit IgGs) (Bio-Rad).

RESULTS

cDNA cloning and determination of the maturation site of cystathionine γ -synthase from *A. thaliana*

A full-length cDNA coding for cystathionine γ -synthase was amplified from an *A. thaliana* cDNA library by using a primer specific for the 3'-end of the *AtcysI* gene [12]. The nucleotide sequence of the *AtCGS* clone (gene database accession number U83500) contained four and six differences from *AtcysI* (accession number X94756) and *CGSI* (accession number U43709) respectively. On the basis of the sequence numbering of the *CGSI* clone [13] these changes were: (1) a C-to-A base change at nt 366, changing the codon from Ile to Pro, and a CAG to AGC change at nt 591–593, changing the codon from Gln to Ser, compared with *AtcysI*; and (2) an A-to-T base change at nt 84, changing the codon from Ser to Gln, a G-to-T silent change at nt 89, a G-to-C silent change at nt 92, a G-to-C base change at nucleotide 226 changing the codon from Gly to Ala, a G-to-C base change at nt 334, changing the codon from Gly to Ala, a C-to-G base change at nt 1448, changing the codon from Ala to Gly, compared with *CGSI*. To verify that these changes were not the result of PCR artifacts or mutations, independent clones amplified by using different thermostable polymerases were isolated and sequenced over the area in question. All the clones contained the changes mentioned above, thus indicating that the differences in cDNA sequences were not due to amplification conditions but most probably resulted from the use of different *A. thaliana* lines to construct the cDNA libraries.

The *AtCGS* nucleotide sequence contains an open reading frame of 1689 bp encoding a predicted protein of 563 residues with a calculated molecular mass of 59850 Da. As noted by Kim and Leustek [13], the 160 N-terminal residues of cystathionine γ -synthase from *A. thaliana* are not present in the bacterial enzyme and share many features with signal sequences required for protein addressing and transport into organelles. The size of the mature subunit was assessed by analysing an *A. thaliana* crude protein extract by Western blotting with antibodies raised against the recombinant protein. This analysis revealed a single polypeptide of approx. 50–55 kDa whose size was difficult to estimate precisely because it appeared as a smiling band, presumably because of gel distortion owing to the large subunit of Rubisco. To define the maturation site of the enzyme, we have purified cystathionine γ -synthase from *A. thaliana* leaves. A crude protein extract was fractionated by anion-exchange chromatography, and fractions containing cystathionine γ -synthase activity were incubated with anti-(cystathionine γ -synthase) IgGs coupled to Protein A-agarose beads. This led to the immunoprecipitation of all the cystathionine γ -synthase activity from the extract. A Western blot analysis of the precipitated fraction revealed two polypeptides of approx. 53 and 50 kDa, which were subjected to Edman degradation. Both polypeptides gave the same N-terminal sequence VRQLSIK, which corresponds to the amino acid sequence (positions 69–75) deduced from the *AtCGS* cDNA. The presence of two polypeptides could be attributed to a degradation of the enzyme at its C-terminal part during the purification procedure, as previously observed during the isolation of cystathionine γ -synthase from spinach chloroplasts [9]. Thus *A. thaliana* cystathionine γ -synthase is synthesized with an N-terminal presequence of 68 residues and the mature form has a predicted molecular mass of 52.8 kDa.

Purification of mature cystathionine γ -synthase from *A. thaliana* overexpressed in *E. coli*

The cDNA region coding mature cystathionine γ -synthase was

Table 1 Purification procedure of cystathionine γ -synthase from *A. thaliana* overexpressed in *E. coli*

Protein was determined with the Bio-Rad protein assay [19] during purification steps or by detection at 205 nm [20] for the pure enzyme. Enzyme activity was determined at 30 °C in the presence of 10 mM OPH and 1 mM cysteine.

	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Crude extract	950	237	0.25	100	—
$[(\text{NH}_4)_2\text{SO}_4]$ fraction	485	218	0.45	92	1.8
Phenyl-Sepharose pool	110	140	1.27	59	5.1
Source Q pool	11	86	7.80	36	31.2
Superdex 200 pool	3	63	21	26	84

Figure 1 Documentation of purification procedure for recombinant cystathionine γ -synthase from *A. thaliana* by SDS/PAGE

Polypeptides were separated on a SDS/PAGE gel [7.5–15% (w/v) polyacrylamide gradient] and stained with Coomassie Brilliant Blue R-250. Lane 1, soluble proteins from *E. coli* BL21(DE3)pLysS cells harbouring the pET MCGS construct (100 μ g); lane 2, $(\text{NH}_4)_2\text{SO}_4$ fraction (100 μ g); lane 3, phenyl-Sepharose CL4B pool (70 μ g); lane 4, Source Q pool (15 μ g); lane 5, Superdex 200 pool (5 μ g); lane 6, molecular mass markers (shown in kDa).

amplified by PCR and cloned in the pET29a(+) expression vector, giving the plasmid pET MCGS. The procedure described in the Materials and methods section allowed us to purify the recombinant enzyme from extracts of *E. coli* BL21 (DE3)pLysS cells harbouring the pET MCGS construct. The procedure consisted of three chromatographic steps and resulted in an overall 84-fold purification with a yield of enzyme activity of approx. 26 %. Thus 3 mg of pure protein was obtained starting from 950 mg of *E. coli* soluble proteins (Table 1). In the presence of 10 mM OPH and 1 mM cysteine, the pure recombinant enzyme exhibited a specific activity of 21 units/mg of protein. For comparison, the natural enzyme purified from spinach chloroplasts had a specific activity of approx. 13 units/mg of protein [9]. The enzyme could be stored at -80 °C for months without noticeable loss of activity. On SDS/PAGE the purified protein migrated as a single polypeptide of 53 kDa (Figure 1), in agreement with the value calculated from the amino acid sequence predicted from the cDNA. A native molecular mass of approx. 200 kDa was determined for recombinant cystathionine γ -synthase by gel-filtration chromatography on Sephacryl S300 (Pharmacia). Thus, as for the natural plant enzyme isolated from spinach chloroplasts [9], the recombinant cystathionine γ -synthase is a tetramer of four identically sized subunits.

Table 2 Substrate specificity of cystathionine γ -synthase from *A. thaliana*

O-substituted derivatives of homoserine were tested at 10 mM in the presence of 1 mM cysteine, and cystathionine was determined by reverse-phase HPLC after O-phthalaldehyde derivatization. Thiol compounds were assayed at 1 mM in the presence of 10 mM OPH and the release of P_i was used to quantify the reactions. Cystathionine γ -synthase activity in the presence of 10 mM OPH and 1 mM cysteine was 21 units/mg.

Substrate	Product	Relative activity (%)
OPH	Cystathionine	100
OSH	Cystathionine	36
OAH	Cystathionine	3
Cysteine	Cystathionine	100
Sodium sulphide	Homocysteine	65
Mercaptoacetic acid	S-Acetylhomocysteine	61
β -Mercaptoethanol	S-Hydroxyethylhomocysteine	10
D,L-Homocysteine	Homolanthionine	5
Cysteine ethyl ester	Cystathionine monoethyl ester	15
Cysteine methyl ester	Cystathionine monomethyl ester	21

Kinetic properties of the recombinant enzyme

The pH dependence of cystathionine γ -synthase activity was determined in Mes, Mops, Hepes, Tricine and glycine buffers from pH 5.0 to 10.5. The optimal pH range was quite narrow with a maximum at pH 7.5, as previously observed for plant cystathionine γ -synthase [9,10]. Steady-state kinetic studies were performed with cysteine as the varied substrate in the presence of various fixed OPH concentrations. The experimental results were consistent with Michaelis–Menten kinetics; parallel lines in the double-reciprocal plots indicated a Ping Pong mechanism for the enzyme reaction. This analysis allowed us to estimate apparent V_{max} and K_m values for cysteine at each concentration of OPH. A saturation behaviour was obtained when these values of V_{max} and K_m were plotted as a function of OPH concentration. Non-linear regression analysis with a hyperbola yielded a V_{max} of 33.6 ± 0.9 units/mg (corresponding to a k_{cat} of approx. 30 s^{-1}) and K_m values of $2.5 \pm 0.2 \text{ mM}$ and $460 \pm 20 \text{ }\mu\text{M}$ for OPH and cysteine respectively (values given as means $\pm$ S.D.).

Several O-substituted derivatives of homoserine and thiol compounds were tested for their ability to substitute for OPH and cysteine respectively (Table 2). Among the three esters of homoserine tested (each at 10 mM), OPH proved to be the more efficient substrate for cystathionine synthesis because γ -replacement of OSH and OAH by cysteine represented only 36 % and 2–3 % of that obtained with OPH respectively. All the thiol compounds tested served as substrates in the γ -replacement of OPH, cysteine giving the highest specific activity (Table 2).

Among these compounds, sodium sulphide and mercaptoacetic acid were efficient substrates because the relative synthetic rates calculated at a fixed concentration of 1 mM were 65 and 61 % respectively, compared with cysteine. Thus the *A. thaliana* cystathionine γ -synthase exhibits a broad specificity for thiol compounds and therefore resembles the *B. sphæricus* enzyme [7]. It must be stressed, however, that direct sulphydration of OPH by sodium sulphide, a reaction that produces homocysteine, is not assumed to participate in the synthesis of methionine *de novo* [23,24]. Indeed, the inability of a cystathionine β -lyase mutant to grow without exogenous methionine definitively rejects this possibility [11].

Because plant cystathionine γ -synthase is at the branch point between methionine and threonine/isoleucine biosynthetic routes, we tested the effect of several intermediates and end-product metabolites belonging to these pathways on enzyme activity. Preincubation of purified cystathionine γ -synthase for 4 min at 30 °C with 10 mM cystathionine, homocysteine, methionine, *S*-adenosylhomocysteine, *S*-methylmethionine, 5-methylthioadenosine, threonine or isoleucine produced no significant change in activity. Under the same assay conditions, SAM produced a weak decrease in cystathionine γ -synthase activity (40 % inhibition at 25 mM). This variation has no physiological significance because the SAM concentration in plant cells is below 50 μ M [25,26] and could presumably be attributed to the amount of sulphate or chloride that is provided by SAM solutions, cystathionine γ -synthase being sensitive to inhibition by a wide variety of salts [9]. We conclude that metabolites linked to methionine and threonine/isoleucine syntheses do not seem to regulate plant cystathionine γ -synthase by feedback inhibition mechanisms.

Interaction between the apoenzyme and the PLP cofactor

Besides the polypeptide absorption at 277 nm, the spectrum of purified recombinant cystathionine γ -synthase showed absorption maxima at 335 and 427 nm. Because a treatment of the enzyme with 10 mM hydroxylamine for 30 min at 30 °C followed by dialysis against 50 mM potassium phosphate, pH 7.5, abolished the 335 and 427 nm bands, both long-wavelength maxima are caused by formation of a Schiff base between the PLP cofactor and a lysine residue of the protein. The 335 nm peak (ϵ 11 100 M⁻¹·cm⁻¹ at pH 7.5) represents the unprotonated form and the 427 nm peak (ϵ 23 700 M⁻¹·cm⁻¹ at pH 7.5) the protonated form of the Schiff base respectively [27]. The stoichiometric ratio of PLP per enzyme subunit was determined by measuring the absorption of the cofactor at 390 nm on exposure of the native holoenzyme to alkaline conditions (0.1 M NaOH). The average PLP content of the holoenzyme was found to be 1 mol/mol of subunit, as observed previously for the bacterial cystathionine γ -synthase [4,5,7].

The holo-cystathionine γ -synthase was converted into the apoenzyme by using the carbonyl reagent hydroxylamine as described above. On removal of the cofactor the apo-cystathionine γ -synthase exhibited no residual activity, and incubation with excess PLP did not result in the recovery of the characteristic 427 nm chromophore and of catalytic activity. Exhaustive dialysis of holo-cystathionine γ -synthase against a 50 mM cysteine/50 mM potassium phosphate (pH 7.5) solution was used as an alternative protocol to release the PLP cofactor from the enzyme. The apoenzyme obtained in this manner regained only approx. 50 % of its original specific activity and absorption at the 427 nm maximum when incubated with excess PLP. These results indicate that some or all the apoenzyme molecules have

		Pyridoxal 5'-phosphate binding site	
		↓	
Pyridoxyl peptide		LGADLVVHSATXYIGGHNDVLAG	
<i>A. thaliana</i>	(368)	LGADLVVHSATKY IGGHNDVLAG	
<i>Z. mays</i>	(314)	LGADLVVHSATKY IAGHNDVIGG	
<i>E. coli</i>	(187)	LGADLVVHSCTKY LNHSDVVAG	
<i>M. liprae</i>	(197)	LGADVVLHSTTKY IGGHSDVVGG	
<i>H. aurantiacus</i>	(187)	LGADMVVLHSTTKY IGGHSDVVGG	
<i>H. influenzae</i>	(189)	LGADVVIHSGTKY IAGHNDALVG	

Figure 2 Determination of the lysine residue involved in PLP binding in cystathionine γ -synthase from *A. thaliana*

The sequence of the trypsin peptide containing the lysine residue involved in PLP binding (modified amino acid designated as X) is aligned with the amino acid sequences of the *A. thaliana* [U83500], *Zea mays* [AF007786], *E. coli* [P00935], *Mycobacterium leprae* [P46807], *Herpetosiphon aurantiacus* [P24602] and *Haemophilus influenzae* [P44502] cystathionine γ -synthases. Conserved residues between the six sequences are in bold. Numbers in parentheses indicate the locations of amino acids within the respective sequences.

been irreversibly denatured or otherwise damaged on the removal of PLP from the holoenzyme.

To determine the lysine residue involved in PLP binding to the *A. thaliana* cystathionine γ -synthase, the purified recombinant enzyme was subjected to chemical modifications involving borohydride reduction and carboxymethylation, and was then digested with trypsin. The pyridoxyl peptide was purified and sequenced on the basis of its fluorescence properties. The sequence obtained showed that the ϵ -amino group of the lysine residue at position 379 formed an internal aldimine with the C-4 formyl moiety of PLP. Multiple sequence alignment of putative or biochemically defined PLP-binding domains of bacterial, viral and plant cystathionine γ -synthases disclosed that binding of the cofactor occurs in a highly conserved motif of 23 amino acid residues (Figure 2).

γ -Elimination of OPH catalysed by plant cystathionine γ -synthase

In the absence of cysteine, cystathionine γ -synthase from enterobacteria catalyses rapid γ -elimination of OSH to give succinate, α -ketobutyrate and ammonia [4,6]. The *A. thaliana* cystathionine γ -synthase was assayed for its ability to produce α -ketobutyrate from OPH by using a coupled assay with NADH and an excess of lactate dehydrogenase. OPH proved to be faintly decomposed via the γ -elimination process: α -ketobutyrate was formed at 7.8×10^{-3} units/mg of protein at 10 mM OPH. Even though this γ -elimination reaction was very weak, it provided an indication that OPH can interact with the free enzyme, thereby supporting the possibility that OPH is the first substrate that is added to the enzyme during the γ -replacement reaction. The velocity of the elimination process is 1/2700 that determined for the physiological γ -replacement reaction in the presence of 10 mM OPH and 1 mM cysteine (21 units/mg). The low rate for the γ -elimination reaction is not restricted to OPH, because the recombinant enzyme supports γ -elimination of OSH (10 mM) with a velocity of 4.6×10^{-3} units/mg. No α -ketobutyrate release could, however, be detected in the presence of 10 mM OAH.

Figure 3 Difference spectra of cystathionine γ -synthase during γ -elimination of OPH

Spectra were recorded in 50 mM Tricine/NaOH, pH 7.5, at 30 °C in the presence of 38 μ M recombinant cystathionine γ -synthase (subunit basis). At zero time, 600 μ M OPH was mixed with the enzyme and spectra were collected sequentially over a 40 min period. Difference spectra were computed as ($\text{scan}_t - \text{scan}_0$) with the following acquisition time points: curve 1, 0 and 40 min; curve 2, 1 min; curve 3, 13 min; curve 4, 17 min; curve 5, 21 min; curve 6, 25 min; curve 7, 29 min.

To understand the mechanism of this huge difference between plant and enterobacterial cystathionine γ -synthases in catalysis of the γ -elimination reaction, the spectral behaviour of the recombinant cystathionine γ -synthase was examined in the presence of OPH. The sequential recording of the enzyme spectrum after the addition of OPH is shown in Figure 3. After the addition of substrate, the 427 nm absorption band characteristic of the enzyme-bound cofactor was rapidly quenched. At 30 °C, this decrease continued for few minutes and then the absorbance gradually returned towards its original value within 40 min. A subsequent addition of OPH yielded the same evolution of the absorption at 427 nm shown in Figure 3, thus confirming the results obtained with the coupled-assay system showing that the OPH substrate added to the enzyme has been consumed. Difference spectra recorded during the course of the γ -elimination reaction also revealed the appearance of a new absorption maximum at 300 nm (Figure 3): A_{300} increased rapidly after OPH addition to the enzyme and gradually returned to its original value. As shown in Figures 4(A) and 4(B), the very rapid quenching of the 427 nm chromophore after the addition of OPH was simultaneous with the appearance of the 300 nm band. As the γ -elimination of OPH progressed, both the absorptions at 427 and 300 nm returned to their initial values with parallel kinetics. This result clearly indicates that both processes are tightly coupled.

Figure 4 Single-wavelength time courses for the γ -elimination and γ -replacement reactions catalysed by cystathionine γ -synthase

Steady-state absorption measurements at 427 nm (A) and 300 nm (B) during the course of γ -elimination of OPH and during transition from γ -elimination to γ -replacement of the phospho group of OPH by cysteine. Data were collected in 50 mM Tricine/NaOH, pH 7.5, at 30 °C in the presence of 19 μ M recombinant cystathionine γ -synthase (subunit basis). At zero time, 25 μ M OPH was mixed with the enzyme and the absorptions at 300 and 427 nm were recorded for 500 s (curve 1). Transition from γ -elimination to γ -replacement reactions was induced by the addition of 25 μ M cysteine 1 min after OPH (25 μ M) mixing with the enzyme (curve 2).

Spectral modifications of the enzyme during the course of the γ -replacement reaction of OPH by cysteine were analysed further. The enzyme was first incubated with OPH alone. When the γ -elimination reaction had developed for a period of time sufficient to provide quenching of the 427 nm chromophore and formation of the 300 nm intermediate, cysteine was added. Figures 4(A) and 4(B) show that the addition of cysteine rapidly led to the recovery of the original 300 and 427 nm bands. This indicates that cystathionine γ -synthase had shifted from the γ -elimination of OPH to the γ -replacement of the phosphoryl substituent of homoserine by cysteine. The γ -replacement reaction is very rapid because OPH and cysteine are consumed within a few seconds.

Inhibition of cystathionine γ -synthase by PAG

PAG has been described as an active-site-directed inhibitor of plant cystathionine γ -synthase [9,10]. Incubation of the recombinant enzyme with micromolar concentrations of PAG produced a time-dependent pseudo-first-order loss of enzyme activity. Activity could not be recovered after gel filtration through Sephadex G25 or after exhaustive dialysis, suggesting a covalent modification of the enzyme. As shown in Figure 5(A), the pseudo-first-order rate constant (k_{obs}) for the inactivation process is saturable. This behaviour indicates that inhibition

Figure 5 Inactivation of cystathionine γ -synthase from *A. thaliana* by PAG

(A) Plot of the pseudo-first-order rate constant for enzyme inactivation as a function of PAG concentration. The curves representing the time course of enzyme inactivation at 25 °C could be fitted by using the equation: remaining enzyme activity (%) = $V_0 e^{-k_{\text{obs}} t}$, where V_0 is the enzyme activity before PAG addition, k_{obs} is the pseudo-first-order rate constant for enzyme inactivation including the excess PAG concentration, and t is the incubation time of the enzyme with the inhibitor. The data representing k_{obs} as a function of PAG concentration were fitted by a non-linear regression analysis to the equation $k_{\text{obs}} = (k_d[\text{PAG}]) / (K_i + [\text{PAG}])$, where k_d is the inactivation rate constant and K_i the apparent inhibition constant, which yielded the best-fit parameters $k_d = 7.2 \times 10^{-3} \text{ s}^{-1}$ and $K_i = 55 \text{ } \mu\text{M}$. (B) Pseudo-first-order rate constant for enzyme inactivation in the presence of PAG and increasing amounts of OPH or cysteine. Enzyme inactivation was conducted at 25 °C in the presence of PAG alone (100 μM) or with the simultaneous addition of OPH (●, 1 μM to 10 mM) or cysteine (○, 0.1–2.5 mM) in the preincubation mixture. k_{obs} values were determined as described above.

follows a two-step mechanism in which the formation of a reversible enzyme–inhibitor encounter complex (characterized by a bimolecular dissociation constant K_i) preceded an irreversible step for inactivation of the enzyme (characterized by a unimolecular process with rate constant k_d). The K_i and k_d values obtained from the data in Figure 5(A) were 55 μM and $7.2 \times 10^{-3} \text{ s}^{-1}$ respectively. These values are quite similar to those previously reported for the wheat and spinach cystathionine γ -synthases [9,10].

The time-dependent inhibition of cystathionine γ -synthase by a fixed PAG concentration of 100 μM was further analysed in the presence of the substrates of the γ -replacement reaction. At high OPH concentrations (10 mM) the enzyme was fully protected against inactivation by PAG (Figure 5B). The pseudo-first-order rate constant for the inactivation process was also decreased by approx. 50 % with 1 μM OPH, a concentration that is 1/2500 the apparent K_m value for OPH. The addition of cysteine (0.1–2.5 mM) during the inactivation process did not offer any

protection of enzyme activity. Again, this finding is consistent with the fact that in the γ -replacement reaction cysteine cannot bind to the enzyme active site without the prior action of OPH.

Because PAG acts as a site-directed inhibitor of cystathionine γ -synthase, we analysed spectral modifications of the enzyme during the course of inactivation. Steady-state analysis after addition of 100 μM PAG to the native enzyme showed a progressive but slight decrease in the characteristic 427 nm band. After a prolonged incubation period at 30 °C resulting in full inactivation of the enzyme, the loss of chromophore absorption was only 10 % of that of the native enzyme. A similar behaviour has been observed for the *S. typhimurium* cystathionine γ -synthase [28]. The proposed inactivation mechanism for this enzyme suggests that the modified enzyme adduct gives visible absorbance that is closely related to the original pyridoxalimine chromophore.

DISCUSSION

Several cDNA clones encoding plant cystathionine γ -synthase have recently been cloned and characterized [12,13]. However, none of them has been yet used to characterize the plant enzyme biochemically. To this end, we have used a reverse transcription–PCR approach with a primer specific to the 3' end of the *Atcys1* gene [12] to isolate a full-length *A. thaliana* cDNA coding cystathionine γ -synthase. With the goal of establishing a heterologous expression system allowing mass isolation of plant cystathionine γ -synthase, preliminary experiments were performed to determine the maturation site for this chloroplastic enzyme. Using polyclonal antibodies raised against the *A. thaliana* cystathionine γ -synthase expressed in *E. coli* as a fusion with maltose-binding protein, we immunoprecipitated the mature native enzyme and determined its N-terminal sequence. As previously observed for spinach chloroplast cystathionine γ -synthase [9], the purified native enzyme from *A. thaliana* migrated on SDS/PAGE as two molecular species of 53 and 50 kDa. Both proteins exhibited the same N-terminal sequence (VRQLSIK). These results indicate that plant cystathionine γ -synthases are highly sensitive to proteolytic degradation at their C-terminal ends.

Heterologous production of the *A. thaliana* cystathionine γ -synthase as a functional mature enzyme was achieved by using a pET expression vector and the corresponding *E. coli* BL21-(DE3)pLysS host strain. We developed a three-step purification procedure that yielded 1 mg of pure enzyme from a 1 litre cell culture with a specific activity of 21 units/mg of protein. For comparison, the purification scheme devised for the isolation of cystathionine γ -synthase from spinach chloroplasts yielded approx. 80 μg of pure enzyme from 20 kg of leaf [9]. Recombinant cystathionine γ -synthase was isolated as the holoenzyme form since a molar ratio of PLP to subunit of 1:1 was determined. Also, we have shown directly that the cofactor is covalently attached to the enzyme via the formation of an aldimine between the carboxy moiety of PLP and the ϵ -amino moiety of lysine at position 379 of the protein precursor. This biochemical characterization confirms that the 23-residue highly conserved region surrounding Lys-379 is part of the active site of cystathionine γ -synthase. This motif is found in the primary sequence of other enzymes involved in the cysteine/methionine biosynthetic pathway that belong to the same class of PLP-dependent proteins and catalyse γ -replacement reactions of C_4 amino acids and β - or γ -cleavage of cystathionine [29]. This observation reinforces the proposal that these enzymes have evolved from a common ancestor and thus explains their relatively broad substrate specificity. In contrast with most PLP-dependent enzymes, in-

cluding cystathionine γ -synthase from *B. sphaericus* [7] and cystathionine β -lyase from *A. thaliana* [30], the apoenzyme prepared from the recombinant cystathionine γ -synthase could not be restored to a fully active holoenzyme on incubation with excess PLP. This marked difference suggests that PLP not only has a catalytic role but might also participate in the conformational organization and stability of the plant cystathionine γ -synthase.

The reaction mechanism of cystathionine γ -synthase in the presence of the physiological substrates for the γ -replacement reaction, OPH and cysteine, was analysed. Steady-state kinetics was consistent with a Ping Pong mechanism. Furthermore the ability of recombinant cystathionine γ -synthase to catalyse the γ -elimination of OPH in the absence of cysteine and the protective effect of OPH against enzyme inactivation by PAG strongly indicate that OPH is the first substrate added to cystathionine γ -synthase during the reaction. The catalytic constant, k_{cat} , for the γ -replacement of OPH by cysteine was 30 s^{-1} , a value that compares well with that determined for the *E. coli* enzyme (12 s^{-1}) [4]. Also, the K_m values for OPH and cysteine (2.5 mM and 460 μM respectively) are similar to those determined for the wheat enzyme [10]. These high values raise the question of cystathionine γ -synthase efficiency *in vivo*. Indeed, the concentrations of OPH and cysteine in the plant cell are 5 and 16 μM respectively, assuming uniform distribution of these compounds [25,31]. Fersht [32] has pointed out that, in contrast with the widely held belief, a low K_m might not be an important component of enzyme catalysis. For example, high K_m values are indicative of high catalytic efficiency because of large k_{cat} values [32]. In support of this finding, we note that the K_d for OPH estimated from the protection experiments against inactivation by PAG was of the order of 1 μM , i.e. approx. 1/2500 the K_m for OPH. This means that the k_{cat} for the γ -replacement reaction is considerably higher than the dissociation rate constant of the enzyme–OPH complex.

Because the enzymes responsible for the synthesis of OPH from aspartate and that utilize OPH as a substrate (cystathionine γ -synthase and threonine synthase; see Scheme 1) are present in the chloroplast [8,9,15], Giovanelli et al. [33] have speculated that the O-phosphoryl substituent of homoserine is confined to this organelle rather than being uniformly distributed within the cell. Thus OPH concentration within the chloroplast could be approx. 50–100 μM [33]. One of the characteristic features of Ping Pong reactions is that the K_m for one of the substrates is dependent on the concentration of the other substrate (Eqn. 1) [34].

$$\frac{v}{V_{\max}} = \frac{[\text{OPH}]}{K_m^{\text{OPH}} + [\text{OPH}] (1 + K_m^{\text{cysteine}}/[\text{cysteine}])}$$

$$= \frac{[\text{cysteine}]}{K_m^{\text{cysteine}} + [\text{cysteine}] (1 + K_m^{\text{OPH}}/[\text{OPH}])} \quad (1)$$

Therefore at a fixed OPH concentration of 50–100 μM the apparent K_m of cystathionine γ -synthase for cysteine would be of the order of 9–17 μM . Because this value is close to the intracellular concentration of this thiol compound, cysteine does not seem to be a limiting factor for the synthesis of cystathionine.

Even though the intraplasmidial OPH concentration could be increased by such an exclusive localization within plastids [33], its estimated concentration would still remain much lower, e.g. by a factor of at least 25, than the K_m value of the plant enzyme for this substrate. Therefore one can calculate from Eqn. (1) that at 50–100 μM OPH and 16 μM cysteine, the cystathionine γ -synthase reaction would proceed only at approx. 1–2 % of its

maximal rate. Thus most of the enzyme seems to be free and does not participate in cystathionine synthesis. We suggest that this might have physiological significance because, in contrast with cystathionine γ -synthase, the K_m of threonine synthase for OPH in the presence of its activator SAM is extremely low, being of the order of 5 μM [14]. For example, it is reasonable to assume that a decrease in the intraplasmidial concentration of SAM will entail an increase in the OPH concentration in this compartment because of a depressed threonine synthase activity. Under these conditions the net rate of cystathionine synthesis will increase because, as stated above, the K_m of this enzyme for OPH is considerably higher than the intraplasmidial OPH concentration. It therefore seems that the kinetic properties of plant threonine synthase (activation by SAM, low K_m for OPH) and cystathionine γ -synthase (high K_m for OPH) are well suited to ensure a rapid adaptation of the partitioning of OPH between the two diverging metabolic pathways in response to fluctuations in the concentrations of OPH and SAM. This interpretation does not exclude other types of regulation of the partitioning between the methionine and threonine pathways. Indeed, methionine or one of its products seems to regulate the expression of cystathionine γ -synthase via repression/depression mechanisms [1].

The recombinant *A. thaliana* enzyme proved able to catalyse a γ -elimination of OPH in the absence of cysteine to produce P, α -aminobutyrate and ammonia. The plant enzyme was, however, considerably less active than the *E. coli* enzyme in this reaction, by a factor of approx. 1500. This huge difference in catalytic efficiency could not be attributed to the nature of the homoserine ester substrate used in the present study, because γ -elimination of the bacterial cystathionine precursor OSH by the plant enzyme was also very slow. In this respect, the *A. thaliana* cystathionine γ -synthase resembles the *B. sphaericus* enzyme, which seems to be much more specific for the γ -replacement than for the γ -elimination of OAH [7]. During the course of the γ -elimination process of OPH, the spectrum of the *A. thaliana* cystathionine γ -synthase undergoes two major modifications. Thus, immediately after OPH addition to the enzyme, the characteristic 427 nm chromophore is quenched and a 300 nm species appears. Then the 300 and 427 nm bands gradually and concomitantly return towards their original values as the OPH substrate is consumed. When the enzyme was incubated with OPH for a period long enough to quench the 427 nm band and to accumulate the 300 nm species, subsequent mixing with cysteine resulted in a very rapid recovery of the original enzyme spectrum. This result indicates that the γ -elimination and γ -replacement reactions have common intermediates and that the addition of cysteine immediately engages the enzyme in the replacement process. Spectral changes of the *E. coli* cystathionine γ -synthase with OSH and cysteine have been recorded by using rapid scanning stopped-flow spectrophotometry, and a reaction mechanism has been proposed for the γ -elimination and γ -replacement processes [35]. The pre-steady-state phase of the γ -elimination reaction is characterized by a rapid decrease in the amplitude of the aldimine chromophore in parallel with the appearance of a 300 nm band. Brzovic et al. [35] have attributed these concomitant evolutions to a six-step process starting with substrate binding to the enzyme cofactor and resulting in the formation of a β , γ -unsaturated ketimine (intermediate XI) after elimination of the γ -substituent (Scheme 2). This reaction intermediate, which is associated with PLP bleaching and appearance of the 300 nm band, corresponds to the last common intermediate in the γ -elimination and γ -replacement reactions catalysed by the *E. coli* cystathionine γ -synthase [35]. It is clear that this intermediate is also detected during γ -elimination of OPH by the *A. thaliana* cystathionine γ -synthase. This finding strongly suggests that the

Scheme 2 Schematic representation of the γ -elimination and γ -replacement reactions catalysed by cystathionine γ -synthase from *A. thaliana*

This simplified scheme was adapted from Brzovic et al. [35]. Only the β,γ -unsaturated ketimine (intermediate XI), the vinylglycine quinonoid (intermediate IV) and the α -aminocrotonate species (intermediate V) are shown. Spectral properties of the original chromophore and the above-mentioned intermediates are indicated. Catalytic steps involving several intermediates are specified by dotted arrows.

mechanism of formation of the β,γ -unsaturated ketimine are the same for the bacterial and plant enzymes, and that a defect in formation of this reaction intermediate was not the cause of the very low activity of γ -elimination encountered with the plant enzyme. It is noteworthy that the γ -elimination reaction with the bacterial enzyme is also characterized by the apparition of a 485 nm species that accumulates with a much slower relaxation time than the β,γ -unsaturated ketimine [35]. This species, which is specific for the γ -elimination reaction, has been assigned by Brzovic et al. [35] to an aminocrotonate derivative of PLP (intermediate V) deriving from the vinylglycine quinonoid species (intermediate IV) by a protonation reaction (Scheme 2). Subsequent transaldimination of intermediate V induces the release of α -iminobutyric acid from the active site, where it undergoes aqueous hydrolysis to yield α -oxobutyrate and ammonia [35]. An analysis of the absorption spectra shown in Figure 3 indicates that the main difference between the γ -elimination reactions catalysed by the plant and bacterial cystathionine γ -synthases is the total absence of the aminocrotonate derivative of PLP from the plant enzyme. This result demonstrates that tautomerization of the β,γ -unsaturated ketimine (intermediate XI) to produce the vinylglycine quinonoid species (intermediate IV) and/or protonation of intermediate IV yielding the aminocrotonate derivative of PLP (intermediate V) are the rate-limiting steps in the γ -elimination of OPH catalysed by *A. thaliana* cystathionine γ -synthase (Scheme 2). Presumably, despite the similarities suggested by the amino acid sequence comparisons in Figure 2, the environments of PLP in the *E. coli* and *A. thaliana* enzymes are different. The recent crystallization of cystathionine γ -synthase from *E. coli* [36] will aid understanding of the differences

between the two active sites. Finally, it is worth noting that the K_m value for cysteine in the γ -replacement reaction differs markedly between the bacterial and plant enzymes. Whereas the former is in the order of 50–70 μ M [4,6], the latter is in the region of 460 μ M (the present study). Thus a low rate of the γ -elimination reaction in plants might prevent consumption of OPH through this futile process, especially in view of the low intracellular cysteine concentration in plants [25].

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