Purification and properties of cystathionine β -lyase from *Arabidopsis thaliana* overexpressed in *Escherichia coli*

Stéphane RAVANEL*, Dominique JOB and Roland DOUCE

Laboratoire Mixte CNRS/Rhône-Poulenc (UMR41), Rhône-Poulenc Agrochimie, 14-20 rue Pierre Baizet, 69263 Lyon cedex 09, France

Cystathionine β -lyase is a key enzyme in sulphur metabolism that catalyses the second reaction specific for methionine biosynthesis, the pyridoxal 5'-phosphate-dependent β -cleavage of cystathionine to produce homocysteine. To obtain insight into the biochemical properties of the plant enzyme, the cDNA encoding cystathionine β -lyase from *Arabidopsis thaliana* was used to construct an overproducing *Escherichia coli* strain. The recombinant enzyme was isolated at high yield (29 mg of pure protein/litre of cell culture) using an efficient two-step purification procedure. Physicochemical properties of the *Arabidopsis* cystathionine β -lyase were similar to those previously reported for the bacterial enzymes. In particular, the native recombinant protein is a tetramer composed of four identical subunits of 46 kDa, each being associated with one molecule of

pyridoxal 5'-phosphate. Interaction between the apoenzyme and pyridoxal 5'-phosphate is extremely tight, being characterized by a K_d value of 0.5 μ M. Purification and sequencing of the phosphopyridoxyl peptide established that Schiff base formation between the cofactor and the holoenzyme occurs at lysine-278. The substrate specificity of the recombinant cystathionine β -lyase resembles that of the enzyme isolated from other sources, cystathionine and djenkolate being the most effective substrates. The cystathionine analogue aminoethoxyvinylglycine irreversibly inactivates the recombinant cystathionine β -lyase. The inactivation is accompanied by dramatic modification of the spectral properties of the enzyme that can be attributed to the attack of the azomethine linkage between pyridoxal 5'-phosphate and lysine-278 of the polypeptide by aminoethoxyvinylglycine.

INTRODUCTION

Cystathionine β -lyase (EC 4.4.1.8) is the second enzyme unique to methionine biosynthesis that catalyses the α,β -elimination of cystathionine to produce homocysteine, pyruvate and NH_3 . In various bacteria including *Escherichia coli*, *Salmonella typhimurium* and *Bordetella avium*, the enzyme has been extensively characterized at both the biochemical and molecular levels [1–6]. The native protein is a homotetramer consisting of 42–45 kDa subunits encoded by the *metC* gene. The holoenzyme contains a firmly bound pyridoxal 5'-phosphate (PLP) prosthetic group that is attached to the protein in a highly conserved region including the lysine residue involved in Schiff base formation [4,7]. The *E. coli* enzyme has been recently crystallized and its three-dimensional structure is being determined [8].

In contrast with bacteria, our present knowledge on the plant enzyme is far more limited. However, there are several reasons why a better characterization of the plant cystathionine β -lyase would be useful. First, unlike the regulatory pattern observed in bacteria [9], methionine seems not only to regulate its own synthesis via feedback inhibition mechanisms but also to control the carbon flux through threonine and the resulting branched-chain amino acids via activation of threonine synthase by *S*-adenosylmethionine [10,11]. Second, because methionine is relatively deficient in legume seeds, a thorough characterization of the regulation processes governing the flux of sulphur between cysteine and methionine is needed to improve nutritional quality of crop plants [12]. Third, cystathionine β -lyase is potentially a suitable target for efficient herbicides, the essential function of this enzyme being exemplified by the isolation of a *Nicotiana plumbaginifolia* Met⁻ mutant defective in cystathionine β -lyase activity and exhibiting an absolute requirement for methionine

for growth [13]. In various plant species, α,β -elimination of cystathionine has been detected in two distinct cell compartments, namely the chloroplast and cytosol [14,15]. Studies on the substrate specificity of the two pools of enzyme activity have allowed us to distinguish between a cystine β -lyase activity located in the cytosol and a chloroplastic cystathionine β -lyase activity [16,17]. Within the chloroplast, the latter enzyme works together with cystathionine γ -synthase, the first enzyme specific for methionine biosynthesis [18], to ensure transfer of the sulphur atom between cysteine and homocysteine. Cystathionine β -lyase has been purified from spinach chloroplasts but, owing to the very low abundance of the enzyme in plant tissues (approx. 0.003 % of the total soluble protein in leaves), only a partial biochemical characterization could be achieved [14]. The recent cloning of an *Arabidopsis thaliana* cDNA encoding cystathionine β -lyase [19] offers the possibility to overexpress the plant enzyme and thereby to investigate its biochemical and structural properties. This cDNA encodes a 50.4 kDa polypeptide that presumably corresponds to an enzyme precursor since the general features of chloroplast transit peptides could be observed in the N-terminal region of the protein [19].

In the present study we describe the cloning of a synthetic gene encoding a functional cystathionine β -lyase from *A. thaliana* into a pET expression vector and the overproduction of this enzyme in *E. coli* cells. We also report a rapid purification procedure allowing the preparation of large amounts of the recombinant protein. This enabled a thorough characterization of the kinetic properties of the enzyme and allowed us to analyse in detail the interaction between the polypeptide and PLP. Finally, we characterized the kinetic parameters of inactivation of the enzyme by aminoethoxyvinylglycine (AVG) and demonstrated that this inhibitor reacts specifically with the PLP groups of cystathionine

Abbreviations used: AVG, L- α -(2-aminoethoxyvinyl)glycine; DTT, dithiothreitol; IPTG, isopropyl β -D-thiogalactoside; NEM, N-ethylmaleimide; PLP, pyridoxal 5'-phosphate; TFA, trifluoroacetic acid.

* To whom correspondence should be addressed.

β -lyase to produce changes in the absorption and fluorescence properties of the bound cofactor.

MATERIALS AND METHODS

Materials

NADH and lactate dehydrogenase were obtained from Boehringer-Mannheim. Cystathionine and monobromobimane were purchased from Calbiochem. 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. Chromatographic columns were from Pharmacia Chemicals except for the Fractogel EMD DEAE 650 (M) ion-exchange column which was purchased from Merck. The pET29a(+) expression vector and the corresponding S-Tag™ purification kit were from Novagen.

Plant material and bacterial strains

A. thaliana (var. *columbia*) plants used for the preparation of total leaf protein extracts were grown in soil for 3 weeks under a 12 h photoperiod of white light from fluorescent tubes ($10\text{--}40\ \mu\text{E}/\text{m}^2$) at 25 °C.

DNA manipulations were performed in *E. coli* DH10B cells (Gibco-BRL) maintained in Luria broth supplemented with carbenicillin (100 $\mu\text{g}/\text{ml}$) or kanamycin (50 $\mu\text{g}/\text{ml}$). Calcium-competent *E. coli* BL21(DE3)pLysS strain (Novagen) was transformed with the pET29a(+) constructions and then grown on Luria broth supplemented with kanamycin (50 $\mu\text{g}/\text{ml}$) and chloramphenicol (25 $\mu\text{g}/\text{ml}$).

Partial purification of cystathionine β -lyase from *A. thaliana* leaves

A. thaliana leaves (1.2 kg) were homogenized in 2.5 litres of extraction buffer containing 0.1 M Tricine/NaOH, pH 7.5, 1 mM DTT, 1 mM EDTA, 50 μM PLP and a mixture of protease inhibitors (5 mM ϵ -aminohexanoic acid and 1 mM benzamidine) using a Waring blender operating at full speed. The homogenate was filtered through muslin, passed through a 20 μm nylon mesh and centrifuged at 18000 *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 % saturation was achieved and the solution was stirred for 30 min at 4 °C. The pellet obtained after a 30 min centrifugation at 18000 *g* was dissolved in the extraction buffer, clarified by centrifugation (48000 *g*, 20 min) and desalted by passage through a Sephadex G25 (M) column (5 cm $\times$ 34 cm) previously equilibrated in buffer A (50 mM Tricine/NaOH, pH 7.5, 1 mM DTT, 1 mM EDTA, 1 mM benzamidine and 5 mM ϵ -aminohexanoic acid). This crude protein extract (7.7 g of protein, 240 ml) was applied to a Fractogel EMD DEAE 650 (M) column (5 $\times$ 22 cm) equilibrated in buffer A. Cystathionine β -lyase activity was eluted with 150 ml of buffer A containing 0.2 M NaCl (flow rate 5 ml/min; fraction size 10 ml). Fractions containing the highest activity were combined and concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (80 % saturation). Proteins were collected by centrifugation (48000 *g*, 20 min), resuspended in buffer A and passed through a Sephadex G25 (M) column (1.6 cm $\times$ 25 cm) equilibrated in buffer A containing 20 % $(\text{NH}_4)_2\text{SO}_4$. This extract (290 mg of protein, 10 ml) was loaded on to an octyl-Sepharose CL4B column (2.6 cm $\times$ 10 cm), previously equilibrated in buffer A supplemented with 20 % $(\text{NH}_4)_2\text{SO}_4$. After the column had been washed with 150 ml of the equilibrating buffer, proteins were eluted with a 600 ml linear

gradient of $(\text{NH}_4)_2\text{SO}_4$ from 20 to 0 % in buffer A (1.5 ml/min, 3 ml fractions). Fractions containing the cystathionine β -lyase activity were pooled, concentrated and desalted in buffer A as previously described. This solution (75 mg of protein, 3.5 ml) was applied to a Mono Q HR 10/10 column (1 cm $\times$ 10 cm; Pharmacia) that had been equilibrated in buffer A. The column was washed with 20 ml of buffer A, and cystathionine β -lyase was eluted with a 150 ml linear gradient of NaCl from 0 to 0.25 M in the equilibrating buffer (flow rate 1 ml/min; fraction size 1.5 ml).

Construction of fusion and mature cystathionine β -lyase expression plasmids

The pET29 F-CBL plasmid coding for a fusion cystathionine β -lyase was constructed by cloning the *HincII*–*XhoI* fragment of the pYES CBL1 cDNA [19] (1306 bp containing the coding region minus the 69 N-terminal residues) into the pET29a(+) vector linearized by *EcoRV* and *XhoI* digestions. This construct encodes a 46 kDa fusion cystathionine β -lyase containing a 30-residue S-Tag pre-sequence that allows rapid affinity purification of the recombinant protein.

The pET29 M-CBL plasmid coding for mature cystathionine β -lyase was constructed via mutagenesis by PCR using the pYES CBL2 cystathionine β -lyase cDNA as a template [19]. The PCR fragment corresponding to the mature cystathionine β -lyase sequence was synthesized by using the following oligonucleotides: P1 (5'-AACTTGGGAGCATATGCAGATCTCAATCGGAAC-3') which introduced an *NdeI* restriction site containing the ATG translation-initiation codon (underlined) directly before the codon corresponding to the first residue of the mature protein (Gln at position 44); P2 (5'-AACCTATGAATTCACAAAAGCTGAGACAAG-3') which is complementary to the 3' end of the cDNA and introduced an *EcoRI* restriction site 30 bp after the stop codon. PCR was conducted for 30 cycles including 1 min of denaturation at 95 °C, 1 min of hybridization at 52 °C and 2 min of DNA elongation driven by the *Pwo* DNA polymerase (Boehringer) at 72 °C. After digestion with *NdeI* and *EcoRI*, the PCR fragment was subcloned into the pET29a(+) vector digested by the same enzymes. This oriented cloning put the cystathionine β -lyase cDNA under the control of the T7 polymerase promoter. The DNA insert was sequenced on both strands [20] to ensure that no mutations had been introduced during the course of the PCR amplification.

Expression and purification of recombinant cystathionine β -lyases

E. coli BL21(DE3)pLysS cells transformed with pET29 F-CBL or pET29 M-CBL were grown at 37 °C in Luria broth supplemented with pyridoxine (10 μM), thiamin (15 μM), kanamycin (50 $\mu\text{g}/\text{ml}$) and chloramphenicol (25 $\mu\text{g}/\text{ml}$) until the A_{600} reached 0.6. Expression of the recombinant proteins was then induced by adding 1 mM IPTG and the culture was maintained at 28 °C for 18 h. Cells were harvested by centrifugation at 2000 *g* for 15 min and resuspension in buffer A. They were then disrupted by sonication for 10 min at 0 °C and lysates were centrifuged at 28000 *g* for 15 min. From this step, two different protocols were developed to purify the fusion (pET29 F-CBL) or the mature (pET29 M-CBL) recombinant cystathionine β -lyases.

The soluble protein extract containing the recombinant cystathionine β -lyase fused to the S-Tag leader sequence was desalted on a PD10 Sephadex G25 (M) column equilibrated in the following buffer: 20 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1 % Triton X-100. This solution was then incubated with S-Protein-Agarose™ in batch processing for 1 h at 25 °C with

gentle shaking to ensure optimal binding of the fusion protein to the matrix. Purification of the mature recombinant protein by cleavage of the leader sequence was achieved with biotinylated thrombin according to the manufacturer's instructions.

The cell-free extract obtained from *E. coli* BL21(DE3)pLysS cells transformed with pET29 M-CBL (940 mg of protein starting from a 1 litre cell culture) was supplemented with 1% (w/v) streptomycin sulphate and the solution was stirred for 15 min at 4 °C. After a 15 min centrifugation at 22000 *g*, solid $(\text{NH}_4)_2\text{SO}_4$ was added to the supernatant with stirring until 80% saturation was achieved. The solution was stirred for 30 min at 4 °C and then centrifuged at 22000 *g* for 20 min. The resulting pellet was resuspended in buffer A and desalted by passage through a Sephadex G25 (M) column (2.6 cm $\times$ 15 cm) equilibrated in the same buffer. This solution (660 mg of protein, 12 ml) was applied to a Fractogel EMD DEAE 650 (M) column (2.6 cm $\times$ 30 cm) previously equilibrated in buffer A. After loading, the column was washed with 200 ml of buffer A and proteins were eluted with a 500 ml linear gradient from 0 to 0.35 M NaCl in buffer A. Cystathionine β -lyase was eluted as a single sharp peak with buffer A containing 160 mM NaCl (4 ml/min, 10 ml fractions). The active fractions were pooled, concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (80% saturation) and resuspended in 6 ml of buffer A. The sample (81 mg of protein) was divided into three equal parts that were applied successively to a Superdex 200 column (1.6 cm $\times$ 60 cm) equilibrated in buffer A supplemented with 150 M NaCl.

Purification of the PLP-binding polypeptide

The mature recombinant cystathionine β -lyase (1 mg in 1 ml) was reduced for 15 min at 0 °C in a buffer containing 50 mM Tricine/NaOH, pH 7.5, and 0.6% (w/v) NaBH₄. 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 borohydride-reduced and carboxymethylated protein was precipitated by addition of 0.1 vol. of 100% trichloroacetic acid. The pellet (15000 *g*, 30 min) was washed twice with 1 ml of cold acetone, dried under air and resuspended in 200 μ l of 70% (v/v) formic acid. The chemical cleavage of the recombinant protein was achieved in the presence of a 100-fold excess of CNBr over protein for 24 h at 25 °C in the dark and under a nitrogen atmosphere. After dilution with distilled water to a concentration of 7% (v/v) formic acid, the peptides resulting from the cleavage were lyophilized and solubilized in 250 μ l of 0.1% (v/v) trifluoroacetic acid (TFA). The pyridoxyl peptide was further purified by reverse-phase HPLC using a μ Bondapak C₁₈ column (3.9 mm $\times$ 300 mm, 10 μ m particle size, 30 nm pore; Millipore-Waters) using 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; solvent B, 0.07% (v/v) TFA in 80% (v/v) acetonitrile. Peptides were eluted from the column in a 90 min linear gradient from 0 to 100% solvent B at a flow rate of 0.75 ml/min. The effluent was monitored by UV absorbance (210 nm) and fluorescence (excitation 330 nm and emission 392 nm) measurements. The purified phosphopyridoxyl peptide was further freeze-dried and sequenced using an Applied Biosystems gas phase sequencer model 494.

Enzyme assay

Cystathionine β -lyase activity was determined using the monobromobimane derivatization procedure [14] or the spectrophotometric assay based on the coupling of pyruvate production to NADH oxidation via lactate dehydrogenase [21]. In both assays, the activity was measured at 30 °C in 50 mM Tris/HCl, pH 8.5, containing 5 mM DTT and 5 mM substrate. For the coupling assay, the reaction mixture (1 ml) also contained 200 μ M NADH and 7 units of lactate dehydrogenase. One unit of activity corresponded to the amount of enzyme that catalysed the formation of 1 μ mol of product/min.

Kinetic and inhibitor studies

To assay for inhibition by SH-blocking agents or phenylglyoxal, purified protein (0.4 μ g) was incubated with various concentrations of the inhibitor for 10 min at 25 °C in 50 mM Tris/HCl, pH 8.5, before the assay of enzyme activity. To test the effect of AVG on cystathionine β -lyase activity, the inactivation reaction was started by the addition of purified enzyme (2.5 μ g) to a 250 μ l solution containing various concentrations of the inhibitor (0–0.4 mM) in 50 mM Tris/HCl, pH 8.5. At various time intervals, aliquots (40 μ l) were removed from the preincubation mixture and assayed for residual cystathionine β -lyase activity. Kinetic and equilibrium data were fitted to the appropriate theoretical equations by using the KaleidaGraph™ program (Abelbeck software).

Electrophoresis and immunoblotting

SDS/PAGE was performed at room temperature in slab gels containing a linear 7.5–15% (w/v) acrylamide gradient. The experimental conditions for gel preparation, sample solubilization, electrophoresis and gel staining were as detailed by Chua [22]. Polypeptides were also transferred electrophoretically onto nitrocellulose sheets (Bio-Rad) as described by Towbin et al. [23] using a trans Blot Cell (semidry system; Bio-Rad). Identification of the cystathionine β -lyase polypeptide was performed using rabbit polyclonal antibodies raised against the recombinant protein followed by detection with goat horseradish peroxidase-coupled anti-rabbit IgGs (Bio-Rad) using 4-chloro-1-naphthol as substrate.

N-Terminal sequence analysis

Partially purified cystathionine β -lyase from *A. thaliana* leaves and recombinant enzymes were separated by SDS/PAGE and transferred electrophoretically to a Pro-Blot protein sequencing membrane (Applied Biosystems) [24]. Polypeptides were visualized by staining with Coomassie Blue R-250 [0.1% (w/v) in 50% (v/v) methanol] for 1 min and destaining with several changes of 50% (v/v) methanol. The stained protein bands of interest were cut out from the membrane and subjected to Edman degradation using a model 494 gas-liquid-phase protein sequencer (Applied Biosystems).

Spectroscopic determinations

Absorption spectra were recorded at 25 °C in 1 cm optical-path-length quartz cuvettes (1 ml) using a Uvikon 860 (Kontron) spectrophotometer. Fluorescence spectra were obtained at 25 °C with a SFM25 (Kontron) fluorimeter using 1 cm optical-path-length quartz cuvettes (200 μ l). Data from spectroscopic determinations were fitted using the appropriate equations by

non-linear regression analysis with the KaleidaGraph program (Abelbeck software).

Protein determination

Protein was measured either by the method of Bradford [25] using Bio-Rad protein assay reagent with bovine γ -globulin as standard or by measuring A_{205} [26].

RESULTS

Immunological characterization and partial purification of cystathionine β -lyase from *A. thaliana*

We have recently reported that a single cystathionine β -lyase activity located in the stromal space of chloroplasts is involved in the *de novo* synthesis of methionine [17]. The isolation of a unique cDNA encoding cystathionine β -lyase in *A. thaliana* confirmed the subcellular distribution of this enzyme since the encoded polypeptide (50.4 kDa) possesses an N-terminal extension that has the structural features of chloroplastic transit peptides [19]. To identify the maturation site of the cystathionine β -lyase precursor, we first purified a recombinant cystathionine β -lyase that was overproduced as a fusion protein in *E. coli* BL21(DE3)pLysS cells using a pET29a(+) vector (pET29 F-CBL). The expression of the recombinant protein proved to be very efficient since cystathionine β -lyase fused to the S.Tag pre-sequence was produced at levels ranging from 10 to 15 % of total soluble bacterial proteins. Therefore, starting from a 100 ml cell culture and using the one-step purification procedure described in the Materials and methods section, about 5 mg of the recombinant cystathionine β -lyase devoid of the leader pre-sequence could be purified. On the basis of SDS/PAGE, the enzyme migrated as a single 44 kDa polypeptide that gave a single N-terminal sequence corresponding exactly to the amino acid sequence deduced from the pET29 F-CBL construction. Rabbit polyclonal antibodies raised against the recombinant enzyme reacted only with a 46 kDa polypeptide in a crude soluble protein extract from *A. thaliana* leaves separated by SDS/PAGE and electrotransferred to nitrocellulose (Figure 1).

Figure 1 Documentation of purification procedure for *A. thaliana* leaf cystathionine β -lyase by SDS/PAGE

Polypeptides were separated by SDS/PAGE and stained with Coomassie Brilliant Blue R-250 (lanes 1–3) or analysed by Western blotting using polyclonal antibodies raised against recombinant cystathionine β -lyase (lanes 4 and 5). Lane 1, molecular-mass markers (in kDa); lanes 2 and 4, crude extract from *A. thaliana* leaf (100 μ g and 250 μ g respectively); lanes 3 and 5, Mono Q HR 10/10 pool (50 μ g and 20 μ g respectively).

The difference in molecular masses between the polypeptide encoded by the full-length cDNA (50.4 kDa) [19] and the enzyme detected by Western blotting in the leaves (46 kDa) clearly demonstrates that cystathionine β -lyase from *A. thaliana* is a chloroplastic enzyme that is synthesized as a precursor and further imported within the chloroplast to yield a mature form. This result is reinforced by the strong cross-reactivity of the antibodies towards cystathionine β -lyase purified from spinach chloroplasts [14].

In order to identify the N-terminal sequence of the mature cystathionine β -lyase, we purified the enzyme from *A. thaliana* leaves. As expected from previous work carried out on spinach leaves [14], cystathionine β -lyase was present in low abundance in *Arabidopsis* and therefore only partial purification of the protein could be achieved. The protein fraction obtained after a three-step purification procedure including anion exchange and hydrophobic chromatography was analysed by SDS/PAGE and cystathionine β -lyase could be identified as an isolated 46 kDa band by Western blotting (Figure 1). This polypeptide was transferred electrophoretically to a protein-sequencing membrane and submitted to automated Edman degradation. Unfortunately, the sequence of the mature enzyme could not be determined because the N-terminal residue of the protein was blocked. Since the N-terminal sequence of other polypeptides transferred to the membrane could be determined, the failure to identify the maturation site of cystathionine β -lyase could not be ascribed to the experimental procedure but rather to a naturally occurring process. The most usual amino acid modifications that preclude Edman degradation are methionine formylation and glutamine cyclization to form pyroglutamate. We note that a glutamine residue is present at position 44 of the cystathionine β -lyase precursor [19] and therefore maturation of the polypeptide at this site would lead to a 45.7 kDa subunit, a molecular mass that compares well with that of the mature enzyme estimated by Western blot. These findings strongly suggest that the Gln-44 residue is the N-terminal residue of the mature chloroplastic cystathionine β -lyase from *A. thaliana*.

Purification of mature chloroplastic cystathionine β -lyase from *A. thaliana* overexpressed in *E. coli*

To express cystathionine β -lyase from *Arabidopsis* chloroplasts in *E. coli*, a synthetic gene coding for the mature form of the protein was obtained by PCR and cloned in the pET29a(+) vector (pET29 M-CBL) as described in the Materials and methods section. Efficient expression of the recombinant protein was obtained in the *E. coli* BL21(DE3)pLysS strain, which possesses the T7 RNA polymerase system but is deficient in two major bacterial proteases [27]. To produce holo-(cystathionine β -lyase), i.e. the PLP-loaded enzyme, in the host cell, the culture medium was supplemented with pyridoxine (precursor of PLP) and thiamin (to bypass the inhibition of this biosynthetic pathway by vitamin B₆), as previously reported for the overproduction of spinach chloroplast *O*-acetylserine(thiol) lyase [28]. As observed by SDS/PAGE, a 46 kDa polypeptide accumulated in the IPTG-induced *E. coli* cells transformed with the pET29 M-CBL construction (Figure 2). This polypeptide, which accounted for approx. 10 % of the bacterial soluble proteins, was assigned to *A. thaliana* cystathionine β -lyase by Western blotting.

A two-step procedure was devised to purify the recombinant cystathionine β -lyase from an *E. coli* cell-free extract. The procedure consisted of anion-exchange chromatography followed by gel filtration, and resulted in an overall 12-fold purification with a yield of enzyme activity of about 76 %. Thus 29 mg of pure cystathionine β -lyase was obtained from 1 litre of

Figure 2 SDS/PAGE analysis of cystathionine β -lyase from *A. thaliana* at various steps of purification from overproducing *E. coli* cells

Polypeptides were separated by SDS/PAGE [7.5–15% (w/v) acrylamide gradient] and stained with Coomassie Brilliant Blue R-250. Lane 1, soluble proteins from *E. coli* BL21(DE3)pLysS cells before induction by IPTG (100 μ g); lane 2, soluble proteins from *E. coli* BL21(DE3)pLysS cells after an 18 h induction by IPTG (100 μ g); lane 3, $(\text{NH}_4)_2\text{SO}_4$ fraction (100 μ g); lane 4, EMD DEAE 650 (M) pool (22 μ g); lane 5, Superdex 200 pool (5 μ g); lane 6, molecular-mass markers (in kDa).

Table 1 Purification of cystathionine β -lyase from *A. thaliana* overexpressed in *E. coli* cells

At each step of the purification, cystathionine β -lyase activity was determined on desalted protein samples in the presence of 5 mM cystathionine. The purification procedure refers to a 1 litre cell culture induced with 1 mM IPTG for 18 h at 28 °C. The values in parentheses were obtained when protein concentration was determined by measuring A_{205} [26] rather than the method of Bradford [25].

	Total protein (mg)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)	Purification (-fold)
Crude extract	940	1880	2.0	100	—
$(\text{NH}_4)_2\text{SO}_4$ fraction	660	1730	2.6	92	1.3
EMD DEAE 650 (M) pool	81	1520	18.8	81	9.4
Superdex 200 pool	58 (29)	1420	24.5 (49)	76	12.2

cell culture (Table 1). On SDS/PAGE, the enzyme preparation migrated as a single 46 kDa polypeptide (Figure 2). As determined by gel filtration on Superdex 200, an apparent molecular mass of 170 kDa was calculated for the native enzyme. Therefore these results indicate that, as previously observed for the spinach chloroplast enzyme [14], cystathionine β -lyase from *Arabidopsis* overproduced in *E. coli* is a tetramer composed of four identical subunits.

Kinetic properties of the recombinant enzyme

In the presence of a saturating concentration of cystathionine (5 mM), the pure overexpressed enzyme exhibited a specific activity of 49 units/mg of protein, a value that compares well with cystathionine β -lyases purified from other sources [2,3,5,14].

Table 2 Substrate specificity of cystathionine β -lyase from *A. thaliana* overexpressed in *E. coli*

Enzyme activity was determined at 30 °C using both the monobromobimane derivatization procedure [14] and the spectrophotometric assay [21]. Kinetic parameters were determined by non-linear regression analysis of the data to the Michaelis–Menten equation. nd, No detectable activity for substrate concentrations ranging from 0 to 30 mM.

Substrate	K_m (mM)	V_{max} (units/mg)
Cystathionine	0.22	51.9
Djenkolate	0.22	42.4
Cystine	0.75	8.3
Lanthionine	18.7	3.0
Homocystine	—	nd
Methionine	—	nd

Furthermore the recombinant enzyme could be stored at -80 °C for several months without any appreciable loss of activity.

Cystathionine β -lyase activity was determined as a function of pH by buffering the reaction mixture with 0.1 M Tris/ KH_2PO_4 or $\text{K}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ from pH 6.0 to pH 9.5 at 30 °C. The recombinant enzyme showed a bell-shaped pH–rate profile with an optimum at pH 8.5. This value is similar to that determined for cystathionine β -lyase isolated from spinach chloroplasts [14], demonstrating that the enzyme would be active in the illuminated stroma.

Several sulphur-containing amino acids were assayed as potential substrates for the enzyme. Table 2 shows that cystathionine β -lyase has a relatively broad specificity towards sulphur-containing amino acids. At a fixed concentration of 5 mM, cystathionine and djenkolate were the most effective substrates for the enzyme. Cystine and lanthionine, to a lesser extent, also acted as substrates whereas no activity could be detected with homocysteine and methionine (Table 2). The recombinant cystathionine β -lyase obeyed Michaelis–Menten kinetics with the four substrates examined (cystathionine, djenkolate, cystine and lanthionine). The apparent K_m values for cystathionine and djenkolate were similar (0.22 mM) whereas the V_{max} for the latter was 80% of that determined for the natural substrate of the enzyme (Table 2). Cystathionine β -lyase from *Arabidopsis* also catalysed the α,β -elimination of cystine to produce cysteine persulphide, pyruvate and NH_3 with a K_m value of 0.75 mM. However, the V_{max} value for this reaction accounted for only 16% of that measured in the presence of cystathionine (Table 2). Finally, lanthionine proved to be a very poor substrate for the enzyme, exhibiting a K_m value above 18 mM and a maximal velocity of only 6% of the maximal value obtained with cystathionine (Table 2).

The effect of temperature on enzyme activity was also investigated in the presence of cystathionine, djenkolate and cystine as substrates. In all cases, enzyme activity increased with temperature until maximal rates were achieved at 60 °C. Above this optimal temperature the activity rapidly decreased so that only 17% of the maximal velocity was detected at 90 °C. The activation energies calculated from Arrhenius relationships in the 10–60 °C interval were 66.1, 76.8 and 52.4 kJ/mol for cystathionine, djenkolate and cystine respectively. The thermal stability of the enzyme was further examined by incubating the samples (8 μ g/ml) in 50 mM Tris/HCl (pH 8.5)/1 mM DTT for 5 min at various temperatures (10–90 °C) before assay. Heat inactivation of cystathionine β -lyase was observed for preincubation temperatures above 50 °C and the enzyme

Figure 3 Titration of the apo-(cystathionine β -lyase) with PLP

(A) Spectral determination of the interaction between the apoenzyme and the cofactor. The apo-(cystathionine β -lyase) (4.8 μ M enzyme subunit) obtained after hydroxylamine treatment was incubated with various PLP concentrations for 15 min at 25 °C before determination of the A_{423} variations. The points are experimental and the continuous line is the best fit obtained after non-linear regression analysis of the data to eqn. (1) in the text. This analysis yielded a K_d of 0.5 μ M. (B) Relationship between the catalytic activity of the enzyme and the increase in A_{423} . The straight line was obtained by linear regression analysis of the data (correlation coefficient, $r^2 = 0.99$).

exhibited only 10 % residual activity after being heated at 60 °C for 15 min.

Interaction between the apoenzyme and the PLP cofactor

Purified cystathionine β -lyase is yellow as a result of the presence of the PLP cofactor bound to the enzyme. At pH 7.5, the native enzyme exhibits absorption maxima at 279 and 423 nm. The peak at the longer wavelength is typical of PLP-dependent enzymes, and is due to the formation of an azomethine linkage between the cofactor and the ϵ -NH₂ group of a particular lysine residue of the polypeptide [29]. The A_{279}/A_{423} ratio was approx. 3.3 and a molar absorption coefficient of 33 880 M⁻¹·cm⁻¹ at 423 nm was calculated for the holoenzyme at pH 7.5. The presence of the PLP cofactor was also demonstrated by fluorescence spectroscopy since on excitation at 412 nm the emitted fluorescence of the holoenzyme was maximal at 484 nm. The stoichiometric ratio of PLP to enzyme subunit was estimated by measuring the A_{390} of the cofactor released on exposure of the native holoenzyme to alkaline conditions (0.1 M NaOH). By this method, the PLP content of the holoenzyme was found to be 1 mol/mol of subunit, as observed previously for the bacterial cystathionine β -lyases [2,3].

The holo-(cystathionine β -lyase) was completely converted into the apoenzyme by incubation with 10 mM hydroxylamine, pH 7.0, for 30 min at 25 °C followed by dialysis against 50 mM Tricine/NaOH, pH 7.5, for 12 h at 4 °C. After extraction of the cofactor, cystathionine β -lyase exhibited no residual activity and no absorption peak at 423 nm. Titration of the apoenzyme with PLP was carried out at equilibrium by measuring the A_{423} after incubation of the apoprotein with various concentrations of the cofactor at 25 °C for 15 min in 50 mM Tricine/NaOH, pH 7.5. The increase in A_{423} was dependent on the added PLP and a maximum was reached for a cofactor concentration that was very close to the concentration of enzyme subunit in the assay. This result indicates a stoichiometric and tight binding of PLP to the plant enzyme (Figure 3A). Therefore the binding of PLP to cystathionine β -lyase was analysed using a tight-binding hypothesis according to eqn. (1):

$$A_{423} = \Delta A_{\max} \frac{e_0 + l_0 + K_d - \sqrt{[(e_0 + l_0 + K_d)^2 - 4e_0 l_0]}}{2e_0} \quad (1)$$

where e_0 and l_0 are the total concentrations of cystathionine β -lyase subunit and PLP respectively, K_d is the equilibrium dissociation constant for the encounter complex and $\Delta A_{\max}$ represents the absorbance change at 423 nm recorded in the presence of an excess of PLP over apoprotein. A K_d value of 0.5 μ M could be determined from the data reported in Figure 3(A), consistent with the occurrence of a very tight interaction between PLP and the *Arabidopsis* cystathionine β -lyase. The addition of PLP to the apoenzyme fully restored the enzyme activity. Furthermore, from titration analysis of the apoenzyme, the data for enzyme activity versus PLP concentration could be fitted to an equation analogous to eqn. (1). This analysis yielded a K_d value for the PLP-cystathionine β -lyase complex that was identical ($K_d = 0.5 \mu$ M) with that obtained from the spectrophotometric titration performed at 423 nm. A plot of the enzyme activity versus A_{423} after incubation of the apoprotein with various concentrations of PLP yielded a satisfactorily linear relationship (Figure 3B), demonstrating a direct correlation between the catalytic activity of the enzyme and the presence of the PLP cofactor linked to the polypeptide.

All the bacterial cystathionine β -lyases cloned so far show a highly conserved domain in the central part of the polypeptide that contains the lysine residue involved in Schiff base formation with the cofactor [30]. To determine the amino acid involved in the linkage of PLP to the *A. thaliana* cystathionine β -lyase, the purified recombinant enzyme was submitted to chemical modifications involving borohydride reduction and carboxymethylation, and was further cleaved with CNBr. Purification and sequencing of the pyridoxyl peptide showed that the ϵ -NH₂ of the lysine residue at position 278 formed an internal aldimine with the C-4 formyl moiety of PLP. A comparison of the amino acid sequences of the PLP-binding domains of bacterial and plant cystathionine β -lyases revealed that binding of the cofactor occurs in a highly conserved motif of 15 amino acids (Figure 4).

Inhibitor studies

Cystathionine β -lyases from spinach, *B. avium* and *Paracoccus denitrificans* were found to be sensitive to thiol-blocking inhibitors such as *N*-ethylmaleimide (NEM) and iodoacetamide [3,4,21,31]. Both compounds caused a marked and time-dependent inhibition of the *Arabidopsis* enzyme. After a 15 min incubation of the purified cystathionine β -lyase with the inhibitors, IC₅₀ values of 2.5 μ M and 1.5 mM were determined for NEM and iodoacetamide respectively. This result suggests that at least one

Figure 4 Determination of the lysine residue involved in PLP binding in cystathionine β -lyase from *A. thaliana*

The sequence of the CNBr peptide containing the lysine residue involved in PLP binding (modified amino acid designated X) is aligned with the amino acid sequences of *A. thaliana* [19], *E. coli* [2], *B. avium* [3] and *S. typhimurium* [6] cystathionine β -lyases. Conserved residues between the four sequences are in bold. Numbers in parentheses indicate the location of amino acids within the respective sequences.

of the five cysteine residues present in the *A. thaliana* cystathionine β -lyase is located within or near the active site of the enzyme. In contrast, the arginine-blocking reagent phenylglyoxal (up to 20 mM) had no marked effect on enzyme activity, suggesting that no arginine residue is involved in the catalytic function of cystathionine β -lyase from *A. thaliana*.

The cystathionine analogues rhizobitoxine and AVG have been previously described as suicide substrates for plant and bacterial cystathionine β -lyases [14,32,33]. The recombinant enzyme from *Arabidopsis* was also strongly inactivated in the presence of micromolar concentrations of AVG, and the inactivation process followed pseudo-first-order kinetics (Figure 5A). A plot of the pseudo-first-order rate constant (k_{obs}) versus AVG concentration exhibited saturation behaviour (Figure 5B). These results indicate that the inactivation process follows a two-step mechanism in which the irreversible inactivation of the enzyme (characterized by a unimolecular process with rate constant k_d) is preceded by the formation of a reversible enzyme-inhibitor complex (characterized by a bimolecular dissociation constant K_i). The values for the inactivation rate constant ($k_d = 17 \times 10^{-3} \text{ s}^{-1}$) and the equilibrium inhibition constant ($K_i = 160 \mu\text{M}$) determined from the data reported in Figure 5(B) are quite similar to those previously obtained for the spinach chloroplast enzyme [14].

The availability of large amounts of the recombinant protein allowed us to analyse directly the interaction between cystathionine β -lyase and AVG by conducting spectroscopic studies. Figure 6(A) shows that the addition of AVG to cystathionine β -lyase resulted in considerable changes in the visible absorption spectrum of the enzyme. These modifications were characterized by a decrease in A_{423} and the appearance of a new maximum at 341 nm. The intrinsic fluorescence of cystathionine β -lyase was also affected by the addition of AVG since emitted fluorescence was maximal at 380 nm on excitation at 340 nm. These results clearly indicate that AVG reacts with the PLP groups of the enzyme to produce changes in the absorption and fluorescence properties of the bound cofactor. Furthermore gel filtration and acetone precipitation did not affect the absorption and fluorescence properties of the AVG-inactivated enzyme. These observations strongly suggest that, during the reaction of AVG with the enzyme, the inhibitor

Figure 5 Kinetics of inactivation of cystathionine β -lyase from *A. thaliana* by AVG

(A) Time course of enzyme inhibition. The purified enzyme (2.2 nM) was preincubated with increasing amounts of AVG in 50 mM Tris/HCl, pH 8.5: 10 μM (●), 20 μM (○), 50 μM (■), 100 μM (□), 200 μM (▲) and 400 μM (△). Aliquots were withdrawn at various times and assayed for residual cystathionine β -lyase activity. The curves could be fitted to the equation:

$$\text{remaining enzyme activity (\%)} = V_0 e^{-k_{\text{obs}} t}$$

where V_0 is the enzyme activity before AVG addition, k_{obs} the pseudo-first-order rate constant for enzyme inactivation including the excess AVG concentration, and t the incubation time of the enzyme with the inhibitor. (B) Plot of k_{obs} as a function of AVG concentration. The data were fitted by non-linear regression analysis to the equation:

$$k_{\text{obs}} = k_d [\text{AVG}] / (K_i + [\text{AVG}])$$

where k_d is the inactivation rate constant and K_i the apparent inhibition constant, which yielded the best-fit parameters: $k_d = 17 \times 10^{-3} \text{ s}^{-1}$ and $K_i = 160 \mu\text{M}$.

attacks the Schiff base linkage between the cofactor and lysine at position 278 to form a complex that is not released from the protein. The recording of the differential spectra obtained for increasing concentrations of AVG showed two isosbestic points at 281 and 386 nm (Figure 6B), indicating the presence of only two species in the mixture, free cystathionine β -lyase and AVG-complexed enzyme. The plots of the absorbance changes at 341 and 423 nm against the inhibitor concentration (Figure 6C) could be fitted to a hyperbolic relationship such as eqn (2):

$$\Delta A = \frac{\Delta A_{\text{max}} [\text{AVG}]}{K_{\text{app}} + [\text{AVG}]} \quad (2)$$

where ΔA and ΔA_{max} are the absorbance changes at given and infinite AVG concentrations respectively, and K_{app} the equilibrium dissociation constant for cystathionine β -lyase-inhibitor

Figure 6 Spectral characterization of the interaction between cystathionine β -lyase and AVG

(A) Absorption spectra of cystathionine β -lyase from *A. thaliana* in native and AVG-complexed forms. Spectra were recorded in 50 mM Tricine/NaOH (pH 7.5)/1 mM DTT/1 mM EDTA in the presence of 0.4 mg of recombinant enzyme (2.2 μ M, spectrum 1) or 0.4 mg of recombinant enzyme treated with 1 mM AVG for 2 min at 25 $^{\circ}$ C (spectrum 2). (B) Differential spectra of mixtures containing 0.4 mg of cystathionine β -lyase and various concentrations of AVG. Spectra were measured as described previously against a reference cuvette containing 0.4 mg of enzyme. Before spectra recordings, cystathionine β -lyase was incubated for 2 min at 25 $^{\circ}$ C with the following AVG concentrations: 0, 5, 10, 20, 50, 100, 150, 200, 400, 600, 1000 and 1500 μ M. Arrows indicate the direction of the absorbance changes. (C) Absorbance changes at 341 and 423 nm plotted against AVG concentration. The absorbance modifications at 341 nm ($\circ$, liganded versus free enzyme) and at 423 nm ($\square$, free versus liganded enzyme) could be fitted using the equation:

$$\Delta A = \Delta A_{\max} [\text{AVG}] / (K_{\text{app}} + [\text{AVG}])$$

where ΔA and $\Delta A_{\max}$ are the absorbance changes at a given and infinite AVG concentrations respectively. From these data the apparent dissociation constant (K_{app}) for the cystathionine β -lyase-inhibitor complex is 235 μ M.

complex-formation. The K_{app} values calculated from this representation at 341 and 423 nm are 242 μ M and 228 μ M respectively. The average value for K_{app} (235 μ M) compares well with that of 160 μ M determined from the enzyme-inactivation data.

DISCUSSION

In plants, the second enzyme specific for methionine biosynthesis, cystathionine β -lyase, is localized in the stromal space of chloroplasts [17]. Owing to the very low abundance of this protein in plant tissues, only a partial biochemical characterization could be achieved of the purified spinach chloroplast enzyme [14]. The cloning of a full-length cDNA encoding the *A. thaliana* cystathionine β -lyase [19] gave us the opportunity to analyse in detail the properties of the recombinant enzyme overexpressed in *E. coli*. Since cystathionine β -lyase is synthesized as a 50.4 kDa precursor that is processed within the chloroplast to yield the mature enzyme, preliminary experiments were carried out to determine the maturation site of the protein. Using polyclonal antibodies raised against cystathionine β -lyase overproduced in *E. coli* cells as a fusion protein, the mature enzyme from *Arabidopsis* leaf was identified as a 46 kDa polypeptide (Figure 1). Unfortunately, owing to a naturally occurring blocking process, we were unable to define precisely the maturation site by sequencing the enzyme partially purified from an *A. thaliana* leaf extract. However, glutamine at position 44 appeared to be a good candidate for the cleavage of the enzyme

precursor since maturation at this site would give rise to a protein of 45.7 kDa. Also, sequence analysis indicates that the amino acid sequence upstream of Gln-44 is typical of chloroplast transit peptides [19,34]. This residue was therefore used as the N-terminal residue of the recombinant enzyme used for the biochemical investigations.

To achieve a large-scale production of the chloroplastic cystathionine β -lyase, the *Arabidopsis* cDNA coding for a functional enzyme starting at Gln-44 was cloned into a pET expression vector that was used to transform *E. coli* BL21(DE3)pLysS cells. Using the proposed two-step purification procedure, 1 litre of an overnight culture resulted in 29 mg of homogeneous protein with a specific activity of 49 units/mg of protein. For comparison, the purification scheme devised for the isolation of cystathionine β -lyase from spinach chloroplasts yielded less than 100 μ g of pure enzyme starting from 20 kg of spinach leaves [14]. As evidenced from the absorption and fluorescence properties of the protein, cystathionine β -lyase was isolated as the holoenzyme form showing that the addition of pyridoxine to the culture medium provided the bacteria with sufficient amounts of PLP to keep pace with the high rate of synthesis of the recombinant enzyme. We have determined biochemically that the cofactor is covalently bound to the enzyme (1 mol of PLP/subunit) via the formation of an aldimine between the carboxy moiety of PLP and the ϵ -NH₂ moiety of the lysine residue at position 278 of the protein precursor. This particular lysine residue is present in all the cystathionine β -lyases so far sequenced and is surrounded by a highly conserved motif of

approx. 15 amino acids (Figure 4). Thus the present results provide direct biochemical evidence to reinforce the finding that this conserved region is involved in PLP binding. Most of the residues that constitute this motif are also present in the primary sequences of other enzymes involved in methionine metabolism, in particular cystathionine γ -synthase and cystathionine γ -lyase. This observation is in accordance with the finding that these proteins belong to the same family of PLP-dependent enzymes [30] and reinforces the proposal that these enzymes are derived from a common ancestor [35]. We have also shown that the holoenzyme can be rapidly converted into an inactive apoenzyme form using a hydroxylamine treatment that cleaves the Schiff base. This process is reversible since a fully active holo-(cystathionine β -lyase) can be restored from the apoprotein after incubation with PLP free in solution. The interaction between the plant apoenzyme and PLP is characterized by a dissociation constant of $0.5 \mu\text{M}$, as determined from both absorbance and activity measurements. This value is 10 times smaller than that determined for the *E. coli* cystathionine β -lyase [2], demonstrating a very tight binding of the cofactor to the plant enzyme.

When studying the α,β -elimination reactions of sulphur-containing amino acids in plants, determination of the enzyme substrate specificity is crucial since it allows distinction between true cystathionine β -lyase activities involved in the synthesis of methionine and other C-S lyase activities. Thus cystathionine β -lyase (EC 4.4.1.8) supports the cleavage of either cystathionine or djenkolate at high levels and shows low activity towards cystine. Conversely, C-S lyases (EC 4.4.1.-) are characterized by marginal cleavage of cystathionine and efficient synthesis of cysteine persulphide from cystine [17,36]. Recombinant cystathionine β -lyase from *A. thaliana* showed a relatively broad substrate specificity toward sulphur-containing amino acids, as previously observed for the spinach [21], *E. coli* [2], *B. avium* [3] and *S. typhimurium* [5] enzymes. For all these enzymes, the highest rates of β -cleavage activity were obtained with cystathionine and djenkolate whereas cystine and lanthionine proved to be poor substrates [2,14,21]. In the case of cystine, as compared with cystathionine, substrate selectivity does not appear to derive from substrate binding but rather from chemical steps (Table 2). Furthermore, since the activation energy values for cystine and cystathionine were nearly the same, and since both substrates showed similar K_m values, it appears that the binding energy of the additional group in cystathionine is not used to bind the substrate but rather to increase k_{cat} . For lanthionine, substrate discrimination occurred at both the substrate-binding and catalytic steps (Table 2). Finally, no activity could be detected for the *Arabidopsis* recombinant enzyme with either homocystine or methionine as substrate. The present observations are in complete agreement with previous analysis of the substrate specificity of a plant cystathionine β -lyase [21] showing that (i) all active substrates contain a C_β L- α -amino acid moiety, (ii) the nature of the sulphur-containing bridge (thioether, thioacetal or disulphide) influences the rate of the α,β -elimination process and (iii) the length of the second moiety, and therefore the spatial separation of the two carboxy groups of the substrate, has a critical role in the efficiency of the cleavage. Further structural analyses are needed to understand the mechanisms responsible for substrate discrimination by the plant enzyme.

Depending on the source of the enzyme, the sensitivity of cystathionine β -lyase towards various SH-blocking agents is very different. It appears that only the enzymes from spinach [21], *B. avium* [3,4] and *P. denitrificans* [31] are sensitive to inhibition by NEM, whereas cystathionine β -lyases from *E. coli* and *S. typhimurium* [37] are not affected by this compound. NEM

caused a marked inhibition of the recombinant *Arabidopsis* enzyme, an IC_{50} value of $2.5 \mu\text{M}$ being determined. This suggests the involvement of at least one cysteine residue in the catalytic activity of this enzyme. Analysis of the amino acid sequence deduced from the *A. thaliana* cDNA [19] indicates that none of the five cysteine residues present in the recombinant cystathionine β -lyase is conserved within the three bacterial enzymes. This suggests either that a cysteine residue is present in the active site of the plant enzyme but absent from the bacterial PLP-binding pocket or that alkylation of the reactive cysteine(s) modifies indirectly the structure of the plant cystathionine β -lyase active site. Site-directed mutagenesis experiments of the five cysteine residues of the protein are in progress to distinguish between these two possibilities.

The search for specific inhibitors of cystathionine β -lyase is of importance since this enzyme catalyses the second reaction specific for a biosynthetic pathway leading to an essential amino acid, making it a suitable potential target for efficient herbicides. In support of this, the inhibition of cystathionine β -lyase by rhizobitoxine or AVG, which behave as suicide substrates for the plant enzyme, causes growth inhibition and dramatic morphological changes in plants [38]. Although some kinetic data on cystathionine β -lyase inactivation by rhizobitoxine and AVG have been previously reported [14,21,33], there is no information on the strength and nature of the interaction between the enzyme and the inhibitors. The availability of large amounts of the *Arabidopsis* enzyme allowed us to demonstrate that AVG reacts with the polypeptide-bound PLP to produce characteristic changes in the absorption and fluorescence properties of the cofactor. In particular, the bleaching of cystathionine β -lyase characterized by a decrease in A_{423} was accompanied by an increase in A_{341} . This result supports the previous observation that (i) the rate of enzyme inactivation is strongly derepressed in the presence of excess free PLP over inhibitor, (ii) the AVG-inactivated enzyme can be re-activated by incubation with PLP and (iii) PLP free in solution interacts with AVG to form an imine [14]. The equilibrium dissociation constant for the enzyme-inhibitor complex determined from spectral analyses ($K_{app} = 235 \mu\text{M}$) compares well with the inhibition constant ($K_i = 160 \mu\text{M}$) and therefore provides evidence that changes in the spectral properties of the protein are related to the inactivation of cystathionine β -lyase.

In conclusion, the cloning of an *A. thaliana* cDNA encoding cystathionine β -lyase allowed us to overexpress this enzyme in *E. coli*, thus providing large quantities of pure protein that could not be obtained by classical biochemical procedures from plant tissues. The availability of the recombinant cystathionine β -lyase will allow thorough structural characterization leading to a better understanding of the reaction mechanism. Furthermore it is interesting to note that the plant cystathionine β -lyase contains in its C-terminal region a similar sequence to the leucine zipper motif [19]. This suggests that the plant enzyme may engage in some specific interaction with another protein component involved in the synthesis of methionine. In this context, it is worth noting the existence of specific protein-protein interactions between two enzymes involved in cysteine biosynthesis in plants, namely serine acetyltransferase and *O*-acetylserine(thiol) lyase [39]. Crystallographic and site-directed-mutagenesis experiments using the present cystathionine β -lyase will therefore help our understanding of the mechanisms governing regulation of the methionine-biosynthetic pathway in higher plants.

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