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Cloning and Bacterial Expression of the *CYS3* Gene Encoding Cystathionine γ-Lyase of *Saccharomyces cerevisiae* and the Physicochemical and Enzymatic Properties of the Protein

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By screening a yeast genomic library, we isolated and characterized a gene rescuing the cysteine requirement in a "*cys1*" strain of *Saccharomyces cerevisiae*. Except for four residues in the open reading frame composed of 1,182 nucleotides, the DNA sequence was the same as that for the *CYS3* (*CYI1*) gene, encoding cystathionine γ-lyase (EC 4.4.1.1), and isolated previously as a cycloheximide-induced gene (B. Ono, K. Tanaka, K. Naito, C. Heike, S. Shinoda, S. Yamamoto, S. Ohmori, T. Oshima, and A. Toh-e, J. Bacteriol. 174:pp.3339–3347, 1992). *S. cerevisiae* "*cys1*" strains carry two closely linked mutations; one (*cys1*) causes a defect in serine *O*-acetyltransferase (EC 2.3.1.30), and another, designated *cys3*, impairs cystathionine γ-lyase activity. Rescue of the cysteine requirement by the gene encoding cystathionine γ-lyase is consistent with both defects being responsible for the cysteine auxotrophy. In an effort to further determine the physicochemical and enzymatic properties of this enzyme, a coding fragment was cloned into an *Escherichia coli* expression plasmid, and the protein was produced in the bacteria. The induced protein was extracted by sonication and purified to homogeneity through one course of DEAE-cellulose column chromatography. The yield of the protein was approximately 150 mg from cells cultured in 1 liter of L broth. The protein showed molecular weights of approximately 194,000 and 48,000 (for the subunit), suggesting a tetrameric structure. An $s_{20,w}$ value of 8.8 was estimated by centrifugation in a sucrose concentration gradient. No sulfhydryl groups were detected, which is consistent with the absence of cysteine residues in the coding sequence. The isoelectric point was at pH 5.2. The protein showed a number of cystathionine-related activities, i.e., cystathionine β-lyase (EC 4.4.1.8), cystathionine γ-lyase, and cystathionine γ-synthase (EC 4.2.99.9) with L-homoserine as a substrate. In addition, we demonstrated L-homoserine sulfhydrylase (adding H_2S) activity but could find no detectable serine *O*-acetyltransferase activity. In this paper, we compare the enzymatic properties of the protein with those of homologous enzymes previously reported and discuss the possibility that this enzyme has a physiological role as cystathionine β-lyase and cystathionine γ-synthase in addition to its previously described role as cystathionine γ-lyase.

The synthesis of cysteine in *Salmonella typhimurium* is well characterized (26). It is synthesized in two steps, *O*-acetyl-L-serine (OAS) synthesis from serine and acetyl coenzyme A (CoA) and subsequent sulfhydrylation of OAS with H_2S, catalyzed by serine *O*-acetyltransferase (EC 2.3.1.30) and OAS sulfhydrylase (EC 4.2.99.8), respectively. This synthetic pathway is considered to be operative in many other organisms, including *Saccharomyces cerevisiae* (19). In *S. cerevisiae*, the second reaction is catalyzed by the bifunctional enzyme, OAS–*O*-acetyl-L-homoserine (OAH) sulfhydrylase (EC 4.2.99.10), which also catalyzes the direct synthesis of homocysteine by sulfhydrylation of OAH with H_2S (50). This enzyme is known to be the product of the *MET17,25* gene (6, 24) (Fig. 1). Serine *O*-acetyltransferase of *S. typhimurium* has been characterized in detail (20, 26, 27). However, information regarding this enzyme in *S. cerevisiae* is significantly lacking. Another cysteine biosynthetic pathway consists of L-cystathionine (CTT) β-synthase (EC 4.2.1.22) and CTT γ-lyase (EC 4.4.1.1) reactions, referred to as reverse transsulfuration (31, 32, 51) (Fig. 1). In this pathway, homocysteine is converted to cysteine. "*cys1*" and "*cys2*" mutants are of interest, since, with two routes to cysteine biosynthesis, no single defect should result in a requirement for cysteine. Ono et al. (38) showed that "*cys1*" strains carry two mutations: one causes a defect in serine *O*-acetyltransferase (*cys1*) and another, designated *cys3*, impairs CTT γ-lyase activity and leads to an accumulation of CTT. The "*cys2*" mutant also carries two mutations responsible for cysteine auxotrophy: one causes a defect in serine *O*-acetyltransferase (its designation is *cys2*) and another mutation, *cys4*, impairs CTT β-synthase (37).

As there is still a significant lack of information regarding the enzymes involved in cysteine and homocysteine biosynthesis in *S. cerevisiae*, we cloned and characterized a gene that was selected for its ability to rescue the cysteine requirement of a "*cys1*" strain. Our studies show that this gene corresponds to the *CYS3* (*CYI1*) gene previously described by Ono et al. (39). The coding sequence has been cloned into an *Escherichia coli* expression vector, and the product has been purified from bacterial cells and characterized in detail. This report describes the purified protein's physicochemical properties and its activities catalyzing various reactions involved in the metabolism of sulfur-containing amino acids. We discuss differences between our study

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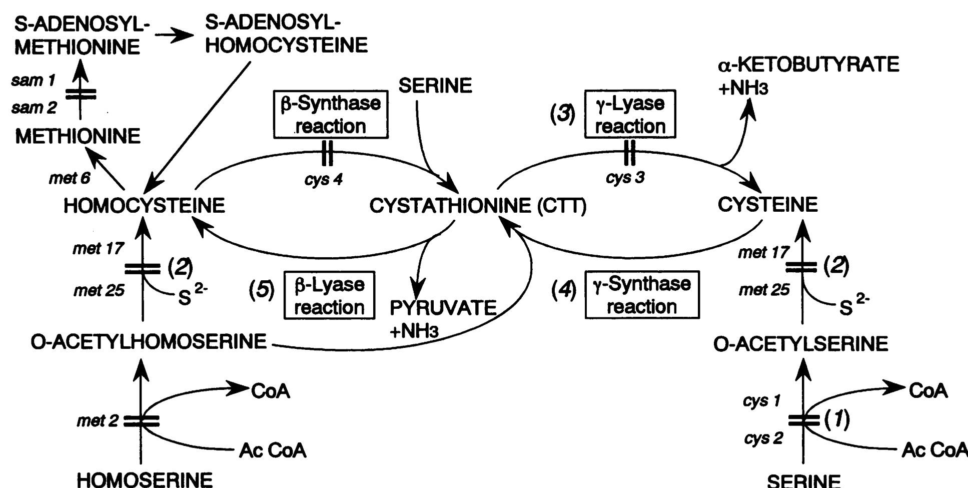

FIG. 1. Metabolism of sulfur-containing amino acids in *S. cerevisiae*. Mutations which have been found are shown ( ), and their names are indicated in italic. CTT-metabolizing reactions are in boxes. Italic numbers in parentheses correspond to those in Table 1.

and previous studies and also speculate about possible in vivo roles of the protein.

MATERIALS AND METHODS

Chemicals and other materials. *O*-Acetylamino acids were synthesized by the method of Nagai and Flavin (35). *O*-Succinyl-L-homoserine (OSH) and other O-substituted derivatives of amino acids were obtained from Sigma Chemical Co. Sephacryl S-300 HR (Pharmacia Fine Chemicals) and DEAE-cellulose (DE-52; Whatman BioSystems Ltd.) were from commercial sources.

Yeast and bacterial strains. The cysteine auxotrophic *S. cerevisiae* strain, GPO6105A (*MAT* **a** *cys1-8 cys3 ura3-52 trp1*), was a gift of Glenn Pure (University of Adelaide). *E. coli* strains used were MC1061 (33), JM101 (52), and HB101 (2).

Media. For *E. coli* strains, the media used were L broth (1% tryptone, 0.5% yeast extract, 1% NaCl [pH 7.0]), *E. coli* minimal medium (34), and 2× YT broth (1.6% tryptone, 1% yeast extract, 1% NaCl [pH 7.6]).

For yeast strains, minimal medium was prepared with 0.17% Difco yeast nitrogen base (without ammonium sulfate and amino acids), 0.5% ammonium sulfate, and 2% glucose. When necessary, supplements were added to the medium at a concentration of 20 μg/ml. Prior to transformation, yeast cells were grown in complete YPD medium (2% peptone, 1% yeast extract, 2% glucose).

Plasmids. The yeast genomic library was prepared and supplied by Marian Carlson (4). The yeast shuttle vector pEMBLYe23 (1) was used for subcloning. Fragments for in vitro transcription were subcloned into pGEM2 (Promega). For expression in *E. coli* cells, the *CYS3* gene was subcloned into an *E. coli* expression vector, pTrc99A (Pharmacia). The constructed plasmids are shown in Fig. 2.

DNA manipulations and nucleotide sequencing. Preparation of plasmid DNA from *E. coli* cultures was performed by the alkaline extraction procedure described by Maniatis et al. (29). All DNA used for yeast transformation experiments was purified by centrifugation at 200,000 × *g* in a cesium chloride density gradient. Restriction endonucleases and other DNA-modifying enzymes were obtained from New England Biolabs, Biotechnology Research Enterprises S. A. Pty. Ltd. (Bresatec), and Takara Shuzo. The nucleotide sequence was determined by the dideoxy chain termination method (42). Deletion fragments generated with *Bal* 31 exonuclease and other suitable fragments for sequencing were cloned into M13mp18 and M13mp19 vectors (52).

Transformations. Transformation of *S. cerevisiae* was performed by the procedure described by Hinnen et al. (16). Otherwise, the lithium chloride technique was employed (18, 40). Transformation of *E. coli* was as described by Maniatis et al. (29).

In vitro transcription, protein synthesis, and tests for serine *O*-acetyltransferase. The DNA fragments containing the *CYS3* open reading frame were cloned into the SP6/T7 transcription vector pGEM2. A resultant clone, pGEM-CYS3, was digested with *Ava*II, which cleaves downstream of *CYS3*, and employed as a template to generate transcripts with SP6 polymerase (Bresatec). In vitro translation of the transcripts was carried out with the rabbit reticulocyte lysate translation system (Amersham, Amersham, United Kingdom). Serine *O*-acetyltransferase activity was checked by observing radioactive conversion of [^{14}C]serine to [^{14}C]OAS in the presence of 1 mM acetyl-CoA after separating the reaction products by thin-layer chromatography as described by Kredich and Tomkins (27).

Subcloning of *CYS3* into pTrc99A. A bacterial expression vector, pTrc99A (Pharmacia), was digested with *Nco*I and *Pst*I so that it could accept a pGEMCYS3-derived fragment (nucleotide 14-686) which encoded the N-terminal segment of the *CYS3*. Nucleotide 1 designates the A of the initiator methionine codon as described previously by Ono et al. (39). The complementary synthetic oligonucleotides (Rikaken Co. Ltd.) encoding the missing NH_2-terminal five amino acids were used as an adaptor (Fig. 2). The constructed plasmid, pTrc-CYSN, was ligated to a *Pst*I-*Ava*II fragment incorporating the remaining C-terminal part of the gene. The *Kpn*I-*Ava*II fragment of pGEMCYS3 (nucleotide 321-1216) was cloned into pTrc-CYSN between the *Kpn*I and the *Pst*I site. The *Ava*II end of the insert and the *Pst*I end of the vector were blunted prior to the *Kpn*I digestion. The resulting plasmid, pTrc-CYS3, was used to overproduce the *CYS3* product.

Purification of the gene product. The *E. coli* cells transformed with pTrc-CYS3 were cultured at 37°C in 1 liter of L

TABLE 1. Summary of assays of enzyme activities

Assay	Enzyme and reaction catalyzed (no. in Fig. 1)	Substance determined (reagent[a] or method)	Reference(s)
1	Serine *O*-acetyltransferase L-Serine + acetyl-CoA → *O*-acetyl-L-serine + CoA (*1*)	CoA (DTNB)	48
2	Sulfhydrylase (adding H_2S) L-Homoserine analog + H_2S → L-homocysteine + substituent (*2*)	Homocysteine (HNO_2)	27
3	Lyase (eliminase) S-amino acid + H_2O → SH compound + α-keto acid + NH_3	SH compound (DTNB)	12
4	Lyase (eliminase) S-amino acid + H_2O → SH compound + α-keto acid + NH_3	α-Keto acid (2,4-DNPH)	13
5	Lyase (eliminase) L-Homoserine analog + H_2O → substituent + α-keto acid + NH_3	α-Keto acid (LDH, NADH)	15
6	CTT γ-lyase CTT + H_2O → L-cysteine + α-ketobutyric acid + NH_3 (*3*)	Cysteine (ninhydrin)	14
7	CTT β-lyase CTT + H_2O → L-homocysteine + pyruvic acid + NH_3 (*5*)	Homocysteine[b] (HNO_2, ninhydrin)	14, 27
8	CTT γ-synthase L-Homoserine analog + L-cysteine → CTT + substituent (*4*)	Cysteine consumed (DTNB)	23

[a] DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); 2,4-DNPH, 2,4-dinitrophenylhydrazine; LDH, lactic dehydrogenase (EC 1.1.1.27).
[b] Amounts of homocysteine produced were calculated as the difference between the amounts of cysteine and total amounts of cysteine and homocysteine.

broth containing ampicillin (50 μg/ml) and 1 mM isopropyl-β-D-thiogalactopyranoside for 28 h by gentle shaking. The cells (5.5 g [wet weight]) were collected by centrifugation at 9,000 × *g* for 10 min and washed with 300 ml of 50 mM Tris-hydrochloride buffer, pH 7.8, containing 30 mM NaCl. The precipitated cells were suspended in 35 ml of 50 mM Tris-hydrochloride buffer (pH 7.8) containing 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 0.6 mM pyridoxal 5′-phosphate (PLP), and 30 mg of lysozyme hydrochloride and then subjected to three cycles of sonication as follows: continuous exposure to sonication at the highest output for 3 min and cooling in ice water for 1 min (ultrasonic processor W-225; Heat Systems-Ultrasonic, Inc.). The clear homogenate obtained was centrifuged at 13,000 × *g* for 15 min. The supernatant fraction (crude extract, 35 ml) was applied to a DEAE-cellulose column (2.6 by 13 cm) equilibrated with 50 mM Tris hydrochloride buffer (pH 7.8) containing 1 mM EDTA, 0.2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM PLP. After washing the column with 200 ml of the same buffer, proteins were eluted at a rate of 0.4 ml/min with 400 ml of an NaCl linear concentration gradient, 0 to 0.25 M, formed in the same buffer. The eluate was fractionated by 4-ml fractions. All the manipulations were carried out at 4°C.

Determination of enzyme activities. All enzymatic reactions were carried out at 30°C with a purified preparation of the gene product as an enzyme and in 100 mM potassium phosphate buffer, pH 8.0, unless otherwise stated. Table 1 summarizes the assay methods employed in this study. PLP was added at a concentration of 0.1 mM in all reaction mixtures except for assay 5, in which the concentration was limited to 0.02 mM in order to avoid disturbance of keto acid determination. One unit of enzyme activity was defined as the amount catalyzing the consumption of 1 μmol of the substrate or the formation of the same amount of the product per min.

Confirmation of products of CTT β- and γ-lyase reactions. Reactions were carried out in 0.5 ml of mixtures containing 100 mM potassium phosphate buffer (pH 8.0), 5 mM CTT, 0.2 mM PLP, and 0.250 mg of the protein for the indicated time. After stopping the reactions with 0.05 ml of 30% trichloroacetic acid, the reaction mixture was treated through a small column of Dowex 50W-X8, and the products were analyzed by high-voltage paper electrophoresis after oxidation with performic acid as described previously (48). Other conditions are described in the legend to Fig. 5.

Checking of CTT γ-synthesis with L-homoserine as a substrate. Reactions were carried out in 2 ml of mixtures containing 100 mM potassium phosphate buffer (pH 8.0), 10 mM OSH (or L-homoserine or OAH), 2.5 mM L-cysteine hydrochloride, 0.2 mM PLP, and an appropriate amount of the protein. Reaction products were treated and analyzed in the same way as described above.

Estimation of molecular weight. Gel filtration was carried out at 4°C on a Sephacryl S-300 HR column (1.5 by 64 cm) equilibrated with 50 mM Tris-hydrochloride (or 100 mM potassium phosphate) buffer (pH 7.8) containing 1 mM EDTA, 100 mM NaCl, 0.2 mM PLP, and 0.05 mM dithiothreitol. Chromatography of the gene product (0.7 mg of protein) and standard proteins was carried out as described previously (48). Gel filtration was also carried out at room temperature by high-pressure liquid chromatography on a TSK gel G3000SW column (0.75 by 30 cm) equilibrated with 50 mM potassium phosphate buffer (pH 7.0) containing 200 mM potassium sulfate. The elution was performed at a flow rate of 0.5 ml/min. The gene product (65 μg) and standard proteins as employed previously (49) (25 to 50 μg) were separately applied to the column. Centrifugation of the product in a sucrose concentration gradient was carried out, by the method of Martin and Ames (30), together with bovine liver catalase and yeast alcohol dehydrogenase, as described previously (47), and the sedimentation coefficient

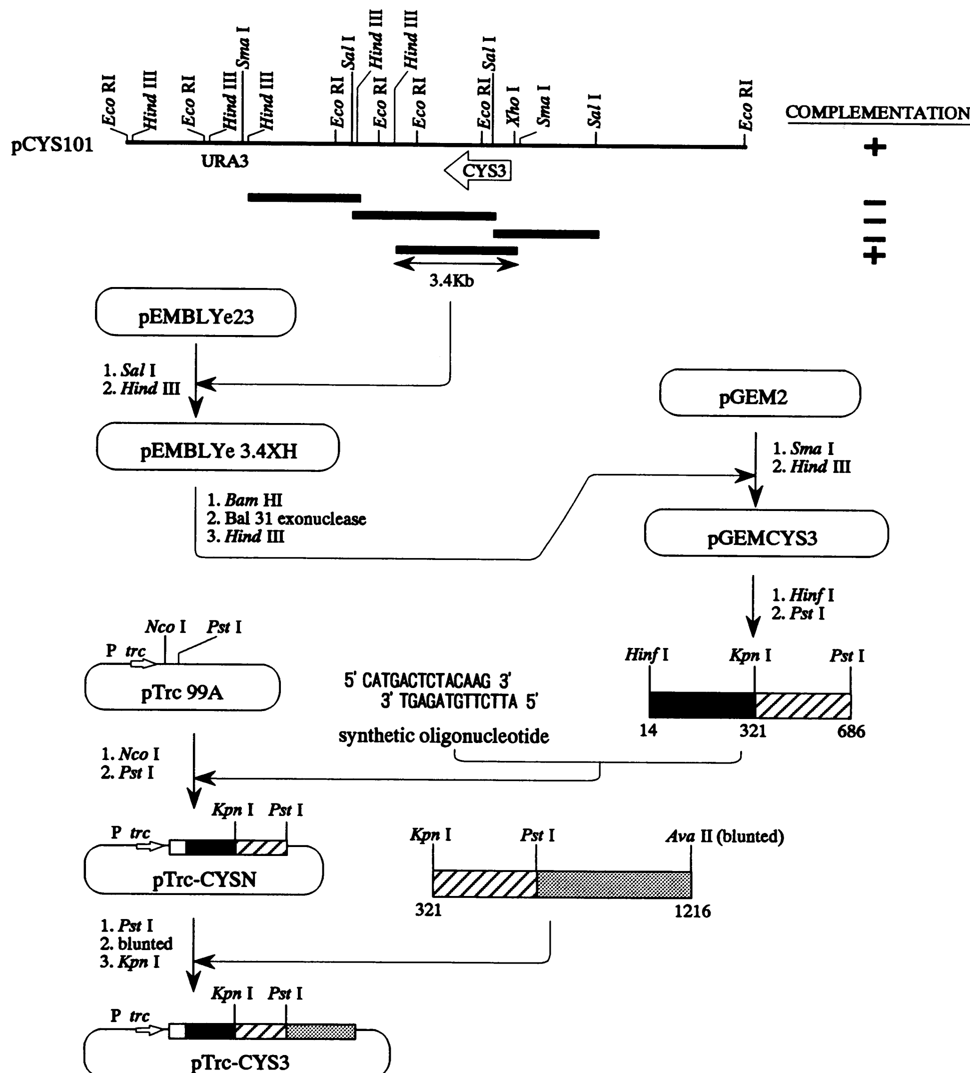

FIG. 2. Localization of the *CYS3* gene and its subcloning into the *E. coli* expression vector pTrc99A. Fragments of plasmid pCYS101 were recloned in pEMBLYe23 (1), and each plasmid was tested for its ability to transform strain GPO6105A to Cys$^+$. +, capacity to transform GPO6105A to Cys$^+$; −, lack of this capacity. A synthetic 14-mer was ligated to the 5′ end of the *CYS3* fragment to generate a product suitable for ligation with *Nco*I-digested pTrc99A.

was calculated on an assumption that the specific volume of the protein was 0.725 (30).

Other procedures. Electrofocusing was carried out to determine the isoelectric point of the gene product, by the method of Vesterberg (45). Polyacrylamide slab gel electrophoresis (PAGE) was carried out with and without sodium dodecyl sulfate (SDS), by the method of Weber and Osborn (46). A molecular weight marker kit (Pharmacia) was employed to calibrate the subunit molecular weight. Content of the sulfhydryl group was determined by the methods of Sakai (41) and of Elmann (10), both under protein-denaturing conditions. Protein was determined by the methods of Lowry et al. (28) and Bradford (3) with bovine serum albumin as a standard.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Data bases with the following accession number: D14135.

RESULTS

Isolation of a clone rescuing the yeast *cys1 cys3* mutation. Plasmid DNA from a yeast genomic library (4) was used to transform strain GPO6105A to the Ura$^+$ Cys$^+$ phenotype (the *URA3* gene was carried on plasmid YEp24, which was used in the library construction). A transformant with a single *URA*$^+$ *CYS*$^+$ plasmid was chosen for further analysis, and the plasmid obtained was referred to as pCYS101. The plasmid pCYS101 contains approximately 10 kb of yeast genomic DNA. A restriction map of the plasmid is shown in Fig. 2.

The two *Sal*I fragments of pCYS101 (3.8 and 3.4 kb), the 3.2-kb *Hin*dIII fragment, and the 3.4-kb *Xho*I-*Hin*dIII fragment were subcloned into the yeast shuttle vector pEMBLYe23 (1) and tested for their abilities to transform strain GPO6105A to Cys$^+$. Among these, only the last plasmid

(pEMBLYe3.4XH) was able to transform GPO6105A to Cys$^+$.

DNA sequencing and homology. The subcloning studies described above indicated that the complementing activity was contained in the insert of pEMBLYe3.4XH spanning the internal *Sal*I site (Fig. 2). The nucleotide sequence of a 2.3-kb region spanning the *Sma*I, *Xho*I, *Sal*I, and *Eco*RI sites has been determined. Analysis of the sequence revealed one major open reading frame of 394 codons starting with an ATG codon located 161 bp downstream of the *Xho*I site. The complete DNA sequence of the open reading frame region was found to be the same as the *CYS3* (*CYI1*) gene recently reported by Ono et al. (39) with only four different nucleotides; nucleotide 130 was C for *CYS3* gene against G for the *CYS3* (*CYI1*) gene, nucleotide 210 was C against G, nucleotide 1,082 was C against G, and nucleotide 1,181 was A against C. As already reported (39), the deduced amino acid sequence of *CYS3* has highly significant homology with several enzymes involved in metabolism of sulfur-containing amino acids; *E. coli* CTT γ-synthase (8), CTT β-lyase (9), rat CTT γ-lyase (11), and *S. cerevisiae* OAS-OAH sulfhydrylase (6, 24). All these proteins are known to have oligomeric structures composed of identical subunits of molecular weights ranging from 40,000 to 50,000 and to require PLP as a cofactor (11, 17, 44, 47). No significant homology was found between *CYS3* and the serine acetyltransferase (*cysE*) gene of *E. coli* (7).

Tests for serine *O*-acetyltransferase activity. As the cloned gene was able to complement the *cys1 cys3* mutation, it was necessary to determine whether the gene encoded a protein with serine *O*-acetyltransferase activity. The gene product was produced in an in vitro translation system as described in Materials and Methods, and the test for the enzyme activity of the product was performed. As a result, no detectable conversion of [^{14}C]serine to [^{14}C]OAS by the catalysis of the gene product was found (data not shown), although the conversion was almost complete with *S. typhimurium cysE* gene product produced in the same way. This result was consistent with the fact that the *CYS3* gene had no significant homology with the gene of serine *O*-acetyltransferase.

Expression of *CYS3* in *E. coli* cells. *E. coli* HB101 cells transformed with the expression vector pTrc99A (culture A) and pTrc-CYS3 (see Fig. 2) (culture B) were cultured in L broth. Cells collected at 3- to 4-h intervals were subjected to SDS-PAGE. Figure 3 shows examples of the result of SDS-PAGE of whole cells of cultures A (Lane V/15) and B (Lanes CYS3/15 and CYS3/28), indicating an abundant expression of the *CYS3* gene product in cells transformed with pTrc-CYS3.

Purity and physicochemical properties of the purified preparation of the protein. As shown in Fig. 3 (lane E), the crude extract of the transformed cells had a very high content of the expressed protein, and specific activities of L-cystine lyase (assay 3) obtained for the crude extract and the purified preparation were 0.086 and 0.316 U/mg of protein, respectively. The protein was eluted from the DEAE-cellulose column with one main peak at fraction 37. Other protein components present in the crude extract are considered to have been removed by washing the column before the chromatography or dispersed in other fractions. Figure 3 also shows the result of SDS-PAGE of the purified preparation of *CYS3* gene product (lane P), indicating that the preparation obtained has a purity of 90% or higher. Electrophoresis without SDS also showed similar purity (data not shown). The high purity of the preparation was also evident

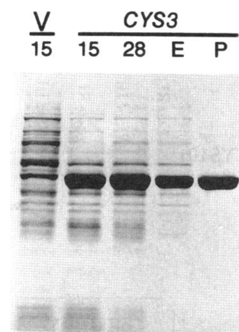

FIG. 3. SDS-PAGE of the *CYS3* gene product. SDS-PAGE was performed with whole transformed bacterial cells, the crude extract, and the purified preparation of the protein. Cells were cultured in 10 ml of L broth containing ampicillin (50 μg/ml) and 1 mM isopropyl-β-D-thiogalactopyranoside. After shaking at 37°C for the indicated time, 1 ml of the culture was centrifuged at 6,000 × *g* for 10 min, and the precipitated cells were subjected to SDS-PAGE. V/15, 15-h-cultured cells transformed with the expression vector (pTrc99A) only; CYS3/15 and CYS3/28, cells transformed with pTrc-CYS3, cultured for 15 and 28 h, respectively. Cell numbers employed in these lanes were approximately the same. E, crude extract; P, purified preparation. Protein amount loaded in these two lanes was approximately 3 μg. Protein was stained with Coomassie brilliant blue R-250.

from the fact that fractions 34 to 41 obtained by the chromatography showed approximately the same specific activity of L-cystine lyase (assay 3) and OSH sulfhydrylase (assay 2) as calculated from Fig. 4A. Approximately 150 mg of protein was recovered in fractions 34 to 41.

Two types of gel filtration were carried out in order to estimate the molecular weight of the gene product as described in Materials and Methods. There was no significant difference in the molecular weight when Tris-hydrochloride buffer was replaced with potassium phosphate buffer, in either system. The averaged value is approximately 194,000. Centrifugation in a sucrose concentration gradient gave an $s_{20,w}$ value of 8.8. SDS-PAGE gave a subunit molecular weight of about 48,000. From these results, the gene product is considered to have a tetrameric structure. Electrofocusing of the protein gave an isoelectric point at pH 5.2. The protein had no sulfhydryl groups even inside the subunit molecule. This is consistent with the gene having no codons for cysteine. These results indicate that the protein purified is the product of the *CYS3* gene.

Enzymatic activities of the gene product. The purified preparation of the gene product showed no serine *O*-acetyltransferase activity in activity assays carried out spectrophotometrically (assay 1). As the *CYS3* gene showed significant (25 to 50%) homology with other enzymes, as mentioned above, we checked whether the protein purified showed those enzyme activities. Several enzyme activities were assayed after both ion-exchange chromatography and gel filtration. Figure 4A shows the elution patterns of protein and two enzyme activities from the DEAE-cellulose column on which the protein was purified. The eluate was concentrated and subjected to gel filtration on a Sephacryl S-300 HR column (Fig. 4B). As clearly seen in the figure, five enzyme activities behaved in parallel with protein, indicating that these activities are catalyzed by one protein corresponding to the gene product.

Lyase (eliminase) activities, with CTT and various sulfur-containing amino acids as substrates, were assayed (assay

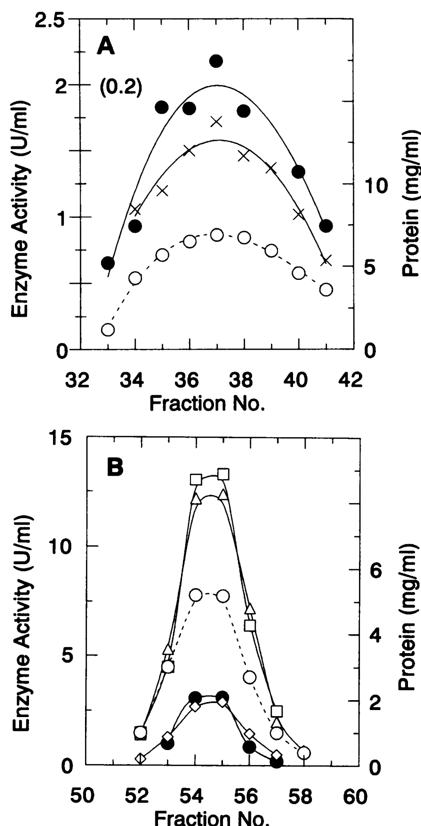

FIG. 4. Elution patterns of protein and some enzyme activities from a DEAE-cellulose column (A) and a Sephacryl S-300 HR column (B). Conditions for the DEAE-cellulose column chromatography (A) are described in Materials and Methods. The eluate (3 ml; 0.5 ml [each] from fractions 34 to 39) was concentrated to 0.9 ml and applied to a Sephacryl column (1.5 by 64 cm) equilibrated with the same buffer as employed in molecular weight estimation. Enzyme activities were determined as described in Materials and Methods. Symbols: ●, cystine lyase (assay 3); ×, OSH sulfhydrylase (assay 2) (activities are shown for the inside scale); □, CTT lyase (assay 3); △, OSH γ-eliminase (assay 5); ◇, CTT γ-synthase (assay 8); ○, protein concentration.

4), and the results obtained are summarized in Table 2. L-Djenkolic acid and L-cystine, in addition to CTT, were more preferable substrates to L-homocystine and other amino acids, so the protein seemed to function more as β-lyase than γ-lyase. The substrate specificity observed is also very similar to that of *E. coli* CTT β-lyase (44). Therefore, the activity with CTT as the substrate was calculated by using the calibration factor for pyruvate (not for α-ketobutyrate) in determination of keto acid produced (assay 4) (13). Figure 5 shows the result of high-voltage paper electrophoresis of products of CTT β- and γ-lyase reactions catalyzed by the protein, confirming the presence of oxidized forms of both homocysteine and cysteine (also see Fig. 6 for the production of homocysteine from CTT synthesized).

Since both β-lyase and γ-lyase activities were detected, comparative determination (assays 6 and 7) of the two activities were performed (Table 2). Kinetic studies revealed that the protein catalyzed the β-lyase and the γ-lyase reactions with K_m values of 0.5 and 0.25 mM, respectively, against the substrate, and V_{max} values of 0.42 and 0.71 U/mg

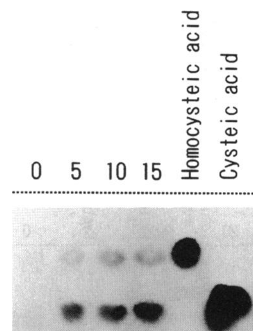

FIG. 5. Confirmation of products of CTT β- and γ-lyase reactions catalyzed by the *CYS3* gene products. Reactions were carried out for the indicated times (minutes) as described in Materials and Methods. The oxidized products were dissolved in 0.3 ml of distilled water. Each 0.06 ml of the solution was spotted at the origin, and the electrophoresis was carried out at pH 1.8 with an electric voltage of 2,000 V for 90 min, as described previously (48). Bottom is anode.

of protein, respectively. These results imply that the protein preferably attacks the bonding between γ-carbon and sulfur. However, this does not rule out that the protein might function as CTT β-lyase as well. The reactivity to CTT as observed for this enzyme preparation has not yet been reported for other enzymes (12, 15, 36, 44).

As seen in Fig. 4B, the protein catalyzed CTT γ-synthesis with OSH and cysteine as substrates. However, this activity is not considered to be functional in vivo, since OSH does not occur naturally in *S. cerevisiae* (39). Given that CTT γ-synthase of *Salmonella* sp. (23) has been reported to

TABLE 2. Catalytic activities of the purified preparation of the *CYS3* gene product

Reaction[a] and substrate	Sp act (U/mg of protein)	Relative activity (%)
Sulfur-containing amino acid lyase activity (assay 4)		
CTT	0.249	100
L-Cystine	0.195	78
L-Homocystine	0.026	10
L-Djenkolic acid	0.425	170
L-Cysteine	0.021	9
DL-Homocysteine	0.014	6
S-Methyl-L-cysteine	0.016	6
S-Carboxymethyl-L-cysteine	0.016	6
CTT lyase activity		
CTT γ-lyase (assay 6)	0.45	100
CTT β-lyase (assay 7)	0.21	47
L-Homoserine analog γ-lyase activity (assay 5 [assay 4])		
OSH	3.20 (3.0)	100 (100)
L-Homoserine	0.35 (0.22)	11 (7)
OAH	0.10 (0.09)	3 (3)
L-Homoserine analog sulfhydrylase activity (assay 2)		
OSH	15.0	100
L-Homoserine	0.82	6
OAH	0.44	3

[a] See Table 1 for assay descriptions. Data for L-homoserine analog γ-lyase activities are for assay 5 and assay 4 (in parentheses).

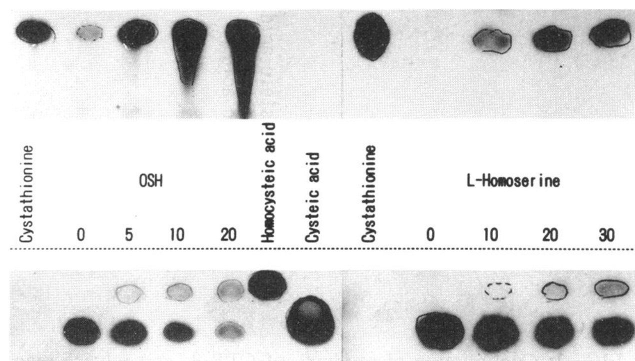

FIG. 6. High-voltage paper electrophoresis of the products of CTT γ-synthase reactions. Reactions were carried out with OSH and L-homoserine as the substrates in 2 ml of the mixture (see Materials and Methods) containing 0.237 and 0.395 mg of the gene product as enzyme, respectively, and stopped by the addition of 0.1 ml of 30% trichloroacetic acid at the indicated time (minutes). The oxidized products were dissolved in 0.3 ml of distilled water and electrophoresed as described in the legend to Fig. 5. Bottom is anode.

catalyze elimination and sulfhydrylation, we checked whether the protein catalyzed these reactions with homoserine analogs as substrates. Table 2 compares the γ-lyase (eliminase) activities of the protein with three amino acids as substrates. Evidently, OSH was the best substrate for the reaction. However, the protein reacted with L-homoserine and OAH as well, although the rate was very low compared with that obtained for OSH. It showed no reactivity with L-methionine, DL-ethionine, *S*-adenosyl-L-methionine, *S*-adenosyl-L-homocysteine, *O*-acetyl-L-tyrosine, and *O*-acetyl-L-threonine and showed very faint reactivity with *O*-phospho-L-serine and L-serine *O*-sulfate.

Sulfhydrylase activity with OSH as a substrate was also evident, as seen in Fig. 4A. However, both L-homoserine and OAH could also be substrates of the protein (Table 2), if a large amount of protein (up to 0.159 mg) was employed. Although the activities found were very low, the *CYS3* gene product was shown to catalyze both γ-elimination and γ-replacement reactions with L-homoserine and OAH as substrates. It must be noted here that L-homoserine was a better substrate in both reactions than OAH. It was relevant to check if the gene product catalyzed CTT γ-synthesis with L-homoserine as a substrate. Figure 6 shows the result of high-voltage paper electrophoresis of the reaction products. As clearly seen in the figure, CTT was synthesized from L-homoserine and L-cysteine in the presence of the gene product. No CTT was observed when OAH was employed in place of OSH, perhaps because of the very low reactivity of the protein to OAH. In addition, homocysteine was also produced when OSH and L-homoserine were substrates. This is considered to be produced from CTT synthesized, on the basis of the CTT β-lyase activity of the same protein (Table 2 and Fig. 5). The amounts of both CTT and homocysteine increased and that of cysteine decreased with an increase in the reaction time.

DISCUSSION

Here we described the characterization of a gene isolated by virtue of its ability to rescue the growth requirement of an *S. cerevisiae* "*cys1*" auxotrophic strain. Sequence analysis indicates that the gene is equivalent to the cycloheximide-inducible gene, *CYS3* (*CYI1*), recently reported (39). This gene was shown to encode CTT γ-lyase and catalyze production of cysteine from CTT. This would be consistent with "*cys1*" strains carrying two mutations (*cys1* and *cys3*), both required for cysteine auxotrophy (38). There is no detectable homology between the amino acid sequences deduced from the *CYS3* gene described here and the *E. coli cysE* gene (7), and we could detect no serine acetyltransferase activity. This suggests that a different structural gene encodes serine acetyltransferase in *S. cerevisiae*, and to date, there is still no information on this enzyme in *S. cerevisiae*. Our results nevertheless confirm that mutation of the *CYS3* gene is essential for the cysteine auxotrophy seen in a "*cys1*" strain. We further characterized the *CYS3* product by using an *E. coli* expression system. Our enzymatic studies revealed that this enzyme catalyzed a number of reactions involved in cysteine biosynthesis.

Ono et al. (39) have described the CTT γ-synthase activity of this enzyme (with OSH as a substrate). They also described its CTT γ-lyase activity and concluded, mainly on the basis of genetic evidence, that its physiological function was in cysteine synthesis from CTT. They further speculated that there would not be a role in CTT γ-synthesis because of the lack of OSH available in the cell. We found that the protein also catalyzed the cystathionine β-lyase reaction and showed that the β-lyase activity was approximately one-half of the γ-lyase activity (Table 2) considerably different from CTT γ-lyases of other organisms (12, 15, 36, 44). This result suggests that further analysis is needed to rule out the function of the protein as CTT β-lyase.

We also studied the substrate specificity of the *CYS3* gene product. The protein reacted with both L-homoserine and OAH, in addition to OSH, giving rise to homocysteine in the sulfhydrylase reactions and CTT in the γ-synthase reactions, although the reactivities were much less than that with OSH (Table 2; Fig. 6). It is interesting that the protein reacted better with L-homoserine than with OAH, while other enzymes catalyzing analogous reactions react with O-substituted derivatives (such as OAH, OSH, or *O*-malonyl-L-homoserine) more than L-homoserine (21, 23, 25). We cannot rule out the possibility that the activity observed with L-homoserine as the substrate functions in vivo, because this substance is readily available in the cell.

Kanzaki et al. (22) have reported CTT γ-synthesis from L-homoserine and L-cysteine, catalyzed by CTT γ-lyase of *Streptomyces phaeochromogenes*. This enzyme catalyzes a γ-lyase (eliminase) reaction with L-homoserine as a substrate but is able to synthesize CTT when L-cysteine is present. The γ-lyase activity is inhibited, and the γ-synthase activity is activated by L-cysteine (highest activation is attained at 10 mM); L-cysteine is considered to be an effector acting on both the allosteric site and the active site of this enzyme. The *CYS3* gene product may be affected in a similar way, because it catalyzed CTT γ-synthesis from L-homoserine at higher cysteine concentration (2.5 mM) (Fig. 6) than at 0.5 mM. Assay 8 could not detect the activity at this concentration (0.5 mM) of L-cysteine. The velocity of the CTT γ-lyase reaction was higher than that of CTT γ-synthesis (with 0.5 mM L-cysteine and 5 mM OSH as substrates) (Fig. 4B; Table 2). In Fig. 6, we can see that large amounts of synthesized CTT remained without being decomposed by CTT γ-lyase activity in the presence of 2.5 mM L-cysteine. This would imply that L-cysteine inhibited the CTT γ-lyase activity of our *CYS3* protein preparation.

Savin and Flavin (43) have reported an unstable CTT

γ-synthase of *S. cerevisiae* which takes OAH and L-cysteine as substrates and is deficient in a *met25* mutant (lacking OAS-OAH sulfhydrylase). One possibility, pointed out by the authors, is that both CTT synthase and OAS-OAH sulfhydrylase are encoded by the same gene. However, a purified preparation of the latter enzyme shows no γ-synthase activity. Another possibility is that this CTT γ-synthase may be encoded by the *CYS3* gene. If this is the case, it would be reasonable to propose that the enzyme may have higher reactivity in vivo with OAH or that the *MET25* gene product is required to form an active *MET25/CYS3* complex which catalyzes CTT γ-synthesis with OAH as a substrate. *Neurospora crassa* CTT γ-synthase has been reported to be composed of two proteins coded for by the *me-3* and *me-7* genes (25). The nucleotide sequence of the latter has been determined (5) and has high homology with the *E. coli metB* gene encoding CTT γ-synthase (8). This association supports the possibility mentioned above.

We have provided a detailed comparative study of the activities of yeast CTT γ-lyase (*CYS3* gene product). Clearly, there are still more studies needed to reach a conclusion regarding the exact physiological role of this enzyme. With further cloning of other enzymes in the cysteine-homocysteine pathway and with more systematic studies of this type, we should edge closer to an understanding of the biochemistry of sulfur metabolism.

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