Cloning, Purification, and Characterization of β-Cystathionase from *Escherichia coli*†

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ABSTRACT: The Clarke–Carbon clone bank of hybrid plasmid *Escherichia coli* DNA has been screened for plasmids able to complement an *E. coli* strain deficient for the production of β-cystathionase. Clone 4-14 had the ability to complement a deletion mutation at this locus and expressed higher levels of β-cystathionase than the wild-type strain. The transfer of the plasmid carried by this clone to a strain that constitutively expresses all the enzymes of the methionine biosynthetic pathway results in 100-fold overproduction of β-cystathionase as compared to wild-type levels. With use of this strain, an efficient three-step purification scheme is described that gives 90% pure enzyme in 54% yield with a specific activity of 215 IU/mg. This enzyme is characterized as to molecular weight (280 000), number of subunits (six), pyridoxal phosphate binding (5.7 mol of pyridoxal phosphate bound/mol of protein, K_m of 0.005 mM), amino acid composition, substrate specificity, and kinetic properties.

Our laboratory has been interested in isolating large quantities of a cysteine and/or a cystine degrading enzyme for the experimental treatment of cysteine-dependent malignant cells (Uren & Lazarus, 1979). β-Cystathionase degrades cystathionine and also cleaves cystine to pyruvate, ammonia, and thiocysteine. The latter is unstable and oxidizes cysteine to cystine with the elimination of hydrogen sulfide (Delavier-Klutchko & Flavin, 1965). In the net reaction both cysteine and cystine can be degraded by the enzyme. β-Cystathionase is an intermediary enzyme in methionine biosynthesis in enteric microorganisms. In *Escherichia coli* various mutations in this pathway that give rise to methionine auxotrophy and their genetic loci have been described (Bachmann et al., 1976).

Clarke & Carbon (1975, 1976) have constructed an *E. coli* clone bank that carries hybrid plasmids of ColE1 and randomly sheared fragments of total *E. coli* DNA. Since these plasmids are under relaxed genetic control, as many as 20 plasmid copies may be present at any given time. Chromosomal genes carried on these hybrid plasmids are therefore amplified over their normal number of gene copies. Gene amplification generally results in gene product overproduction in such strains. Specific plasmid-associated chromosomal genes could be identified by complementation after conjugation between cells of the clone bank and appropriate deficient recipients (Clarke & Carbon, 1976). In order to obtain an overproducing *E. coli* strain as a source for β-cystathionase, we screened the Clarke–Carbon colony bank by conjugational analysis. Our recipient strain carried a deletion in the gene that codes for β-cystathionase. This report describes the selection of a 100-fold overproducer, a rapid efficient purification, and the characterization of this enzyme.

Materials and Methods

L-Cystathionine was purchased from Calbiochem Corp.; L-cystine, L-cysteine, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), iodoacetic acid, pyridoxal phosphate (PLP), and djenkolic acid were from Nutritional Biochemical Corp.; acrylamide, bis(acrylamide), tetramethylethylenediamine, Bio-Gel hydroxylapatite (HTP), and sodium dodecyl sulfate were from Bio-Rad Laboratories; DL-diaminopimelic acid, dithioerythritol (DTE), adenosine, 2,2′-dithiodipyridine, β-cyanoalanine, *S*-adenosylmethionine iodide, *S*-adenosylhomocysteine, and allylglycine were from Sigma Chemical Co.; 3,3,3-trifluoro-DL-alanine was from MCB Reagents; Sephacryl S-300, blue Sepharose CL6B, and molecular weight calibration kit were from Pharmacia Fine Chemicals; EDTA[1] was from Fisher Scientific Co.; guanidine hydrochloride was from Eastman Organic Chemicals Ltd.; DEAE-cellulose (DE-52) was from Whatman Ltd., and L-*meso*-lanthionine and L-homolanthionine were from California Corp. for Biochemical Research. DL-Propargylglycine was synthesized by the methods of Gershon et al. (1954) and DL-2,7-diaminooct-4-ynedioic acid by the methods of Schlogl (1958). All other chemicals used were of analytical reagent grade.

Mating Analysis. The 2112 clones of the Clarke–Carbon collection (obtained from Dan Frankel, Harvard Medical School) were mated with JU100 (a streptomycin-resistant strain of GUC41 obtained from Ronald Greene, Duke University) by replicate plating the library with a 48-pin block onto a lawn of 1×10^7 recipients on 0.2% glucose minimal medium (M63) (Miller, 1972a) containing 0.2 mM threonine and leucine, 0.5 mg/mL streptomycin, 0.01 mg/mL thiamine hydrochloride, 1.5% Difco agar, and 0.05 mL/plate colicin. Colicin was prepared from strain DF466 (obtained from Dan Frankel, Harvard Medical School) by the procedure of Spudich et al. (1970). The plates were grown for 1–2 days, and any colonies that arose were restreaked on the same selection media.

Liquid matings were performed among subclones of JA200/pLC4-14. In these experiments both the donor clones and recipient strain were preincubated in nutrient broth for 3–4 h prior to streaking on selection media. Since the constitutive strain RG100 (obtained from Ronald Greene, Duke University) was F^+, F^- phenocopies were prepared by the procedure of Miller (1972b) prior to mating with the various JA200/pLC4-14 subclones. Exconjugates were selected by streaking on glucose minimal plates containing 0.01 mg/mL thiamine and 0.05 mL/plate colicin. Colonies that arose were restreaked on the same selection media.

Methionine Labeling. By use of the procedure of Neidhardt et al. (1980), strains RG100 and RG100/pLC4-14 were grown

† From the Sidney Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115. *Received November 30, 1981.* This work was supported by U.S. Public Health Service Grants CA-19589-06 and CA-26358-03.

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-3064$01.25/0

overnight in nutrient broth to an optical density of 2.0 at 420 nm. Five-milliliter cultures containing 0.2 mg/mL chloramphenicol were incubated overnight at 37 °C. The cells were harvested, washed, and resuspended in glucose minimal media containing 20 mCi/mL [^{35}S]methionine (New England Nuclear) and incubated for 20 min. The cells were again harvested, washed 5 times, and broken in an Aminco French pressure cell. Extracts were centrifuged and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis (Weber & Osborn, 1969) with a sample of purified β-cystathionase as control. The frozen gels were sliced into 3-mm sections, and each slice was dissolved in 2% NH_4OH–30% H_2O_2, mixed with Biofluor, and counted in a Beckman liquid scintillation counter. The control gel was stained with Coomassie blue, destained, and scanned at 629 nm with a Gilford Model 2520 gel scanner.

Enzyme Assay System. β-Cystathionase was assayed by a modified procedure of Flavin & Slaughter (1971). The reaction mixture in 1 mL contained 0.1 M sodium phosphate buffer, pH 9.0, 2 mM L-cystathionine, 0.2 mM DTNB reagent, and a suitable amount of the enzyme. The enzyme activity was measured with a Gilford spectrophotometer at 412 nm. One enzyme unit represents the formation of 1 μmol of aryl mercaptan/min under standard assay conditions at 37 °C. The protein was determined by the Bio-Rad protein assay (Bradford, 1976) unless otherwise indicated.

E. coli Cells. The RG 100/pLC4-14 cells were grown at 37 °C in 2-L flasks containing 0.8% bacto nutrient broth (Difco Laboratories) sterilized at 15 psig for 20 min. The cells were harvested after 18 h of incubation by centrifugation at 8000*g* for 10 min. After being washed 2 times with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.1 mM DTE and 0.01 mM PLP (buffer A), the cells were stored in the freezer at −20 °C.

Cell Extraction. E. coli cells (18 g, wet wt) were suspended in 360 mL of distilled water, pH 7.0, containing 0.1 mM DTE and 0.01 mM PLP, and the cells were broken in an Aminco French pressure cell at 20 000 psig. The extract was centrifuged at 10000*g* for 15 min, and the supernatant served as the crude extract. All the purification steps were then carried out at 4–10 °C.

DE-52 Cellulose Chromatography. The crude extract was loaded on a 20 × 2.5 cm column of DE-52 cellulose preequilibrated with buffer A. The column was then washed with 250 mL of buffer A and eluted with a 250-mL by 250-mL gradient of 0.05–0.35 M NaCl in buffer A. Ten-milliliter fractions were collected, and those showing over 7.5 units/mL enzyme activity were pooled.

Bio-Gel HTP Adsorption. The pooled fractions from the DE-52 cellulose column were diluted with buffer A to adjust conductivity to 5 mmhos/cm and then treated with 25 g of Bio-Gel HTP preequilibrated with distilled water containing 0.1 mM DTE and 0.01 mM PLP. After 30 min the suspension was centrifuged at 10000*g* for 15 min. The supernatant showed no activity and was discarded. The residue was further suspended in 500 mL of 0.06 M sodium phosphate buffer, pH 7.0, containing 0.1 mM DTE and 0.01 mM PLP. After 30 min the suspension was again centrifuged at 10000*g* for 15 min, and the supernatant was collected.

Blue Sepharose CL6B Chromatography. The eluate from Bio-Gel HTP was loaded on a 75 × 1.7 cm column of blue Sepharose CL6B preequilibrated with buffer A. The column was washed and eluted as described under DE-52 Cellulose Chromatography. Ten-milliliter fractions were collected, and those having specific activity over 200 units/mg of protein were pooled.

For concentration purposes the pooled fractions were further diluted with buffer A to adjust the conductivity to 5 mmhos/cm and loaded on a 5-mL column of DE-52 cellulose preequilibrated with buffer A. The enzyme was then eluted with 0.3 M NaCl in buffer A. One-milliliter fractions were collected, and those showing over 100 units were pooled. This pool provided the concentrated enzyme with a specific activity over 200 units/mg of protein.

Polyacrylamide Gel Electrophoresis. Analytical and sodium dodecyl sulfate ($NaDodSO_4$) gel electrophoresis was carried out as described by Clarke (1964) and Weber & Osborn (1969). The enzyme from the pool after blue Sepharose CL6B chromatography showed primarily a single band on analytical and $NaDodSO_4$–polyacrylamide gels. Area analysis of the scanned gels following Coomassie brilliant blue staining indicated that the enzyme was over 90% pure (see Figure 1). Bovine serum albumin (M_r 67 000), aldolase (subunit M_r 39 500), and lysozyme (M_r 14 000) were used as standard calibration proteins. The subunit molecular weight of the purified β-cystathionase appeared to be about 45 000 from the plots of molecular weight vs. R_f values of the standard proteins.

Molecular Weight of β-Cystathionase. On the basis of a globular shape, the molecular weight of the enzyme was determined on a 50 × 3.5 cm Sephacryl S-300 column. The buffer used during these studies was phosphate-buffered saline (PBS), pH 7.4, containing 1 mM EDTA. The enzyme retained full activity after overnight dialysis against this buffer. The void volume of the column was determined with blue dextran (M_r 2 000 000). Thyroglobulin (M_r 669 000), ferritin (M_r 440 000), catalase (M_r 232 000), aldolase (M_r 158 000), and bovine serum albumin (M_r 67 000) were used as standard calibration proteins. The molecular weight of the purified β-cystathionase was found to be 270 000–290 000 from the plots of K_{av} vs. molecular weight of standard proteins.

Amino Acid Composition of β-Cystathionase. The total amino acid composition of β-cystathionase except for tryptophan was determined with a Beckman Model 121MB microcolumn amino acid analyzer following hydrolysis in 6 N HCl at 110 °C. Amino acid stability was determined by least-squares extrapolation to zero time from hydrolysis times of 24, 48, and 72 h. All amino acid values were within experimental error of the 24-h hydrolysis time. Consequently, the mean of five determinations at 24 h is presented in Table III. Tryptophan residues in the enzyme were determined spectrophotometrically according to the method of Edelhoch (1967). Cysteine residues were determined after reduction and carboxymethylation of the enzyme as described by Hirs (1967). Free SH groups were titrated with DTNB by the method of Ellman (1959). The extinction coefficient at 280 nm for the enzyme was calculated by summing the amino acids recovered from a hydrolysate of a known 280-nm absorbing sample with correction for the cysteine and tryptophan loss during hydrolysis. A 1 mg/mL protein sample had an absorbance of 1.25 by this technique.

Results

β-Cystathionase Cloning. β-Cystathionase is an intermediary enzyme in the methionine anabolic pathway coded for by the *metC* gene. A deletion mutant at this locus that exhibits methionine auxotrophy was originally described by Guterman & Dann (1973) and further characterized by Hunter et al. (1975). This strain, GUC41, was used as a recipient strain for conjugational analysis with the Clarke–Carbon clone bank of hybrid plasmid–chromosomal DNA fragments. The plasmids in this library undergo F^+-mediated transfer at high

Table I: Purification Profile of β-Cystathionase from *E. coli* Cells

fraction	volume (mL)	enzyme act. (units)	total protein (mg)	sp act. (units/mg)	purification (x-fold)	yield (%)
crude extract	365	4216	3504	1.20	1.0	100.0
DE-52 cellulose (pool)	150	3494	480	7.28	6.05	82.9
Bio-Gel HTP (pool)	250	3030	200	15.2	12.6	71.8
blue Sepharose CL6B (pool)	105	2258	10.5	215	179	53.5

frequency, and if they carry the *metC* gene, they should overcome the methionine auxotrophy of the recipient strain. In earlier studies the 2112 clones of the bank were mated with this recipient strain. Selection against the bank was carried out by the lack of tryptophan and against the recipient by the lack of methionine in the selection media. This selection proved not to be stringent enough. Thus double selection against both parents was examined. A streptomycin resistance mutation was added to this recipient strain by P1 transduction (gift of Michael Berman, Frederick Cancer Research Center). The 2112 clones were then remated with this recipient, JU100. Selection against the clone bank was achieved by the lack of tryptophan and the presence of streptomycin while selection against the recipient required a lack of methionine and the presence of colicin. A single clone gave Met^+ Trp^+ colonies under these conditions (clone 4-14). Measurements of the β-cystathionase specific activity in this progeny strain grown in glucose minimal medium containing threonine and leucine demonstrated a 17-fold higher level of this enzyme compared to that of wild-type *E. coli*.

The enzymes of the methionine metabolic pathway are regulated by a diffusible product of the *metJ* gene (Su & Greene, 1971). Mutants at this locus are constitutive for all enzymes of the pathway. Therefore, to further enhance β-cystathionase production, we mated JA200/pLC4-14 subclones with this *metJ* mutant, RG100. Selection against the JA200/pLC4-14 strain was achieved by the lack of threonine and leucine, and selection against RG100 was achieved by the presence of colicin (see Materials and Methods). Approximately 100-fold overproduction of the enzyme was seen when enzyme levels from these RG100/pLC4-14 strains that carry the plasmid *metC* gene are compared to those of JA200/pLC strains that do not carry this genetic locus, and 7-fold overproduction was evident within the same RG100 genetic background for strains containing plasmids with the *metC* locus compared to those without.

Further evidence that β-cystathionase is coded by the plasmid is shown in Figure 1. Following chloramphenicol treatment of plasmid-carrying strains, protein synthesis has been shown to be primarily directed by plasmid gene products (Neidhardt et al., 1980). This figure illustrates that the major newly synthesized gene product in strain RG100/pLC4-14, which is absent from the nonplasmid-containing parent RG100, has the same $NaDodSO_4$–polyacrylamide gel electrophoretic mobility as purified β-cystathionase. These data are consistent with this plasmid-encoded protein being β-cystathionase rather than a stimulator of β-cystathionase activity.

Enzyme Purification. Table I shows the purification scheme for β-cystathionase. The crude cell specific activity of 1.20 IU/mg of protein is 13-fold higher than that of *Salmonella typhimurium* met-A-15, which expresses β-cystathionase 7-fold higher than wild type (Guggenheim, 1971). The enzyme was purified 179-fold with 54% final recovery. The final specific activity achieved was 215 units/mg of protein. This specific activity is about 27-fold higher and the final recovery is about 5-fold higher than that by the best previously reported procedure for *S. typhimurium* β-cystathionase purification (Guggenheim, 1971). The enzyme represents about 0.56% of the total soluble cell protein.

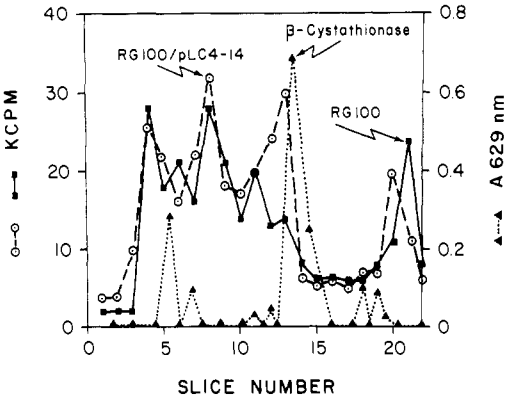

FIGURE 1: $NaDodSO_4$–polyacrylamide gel electrophoresis of purified β-cystathionase isolated from strain RG100/pLC4-14, stained with Coomassie blue (▲), and crude extracts from strains RG100/pLC4-14 (⊙) and RG100 (■) labeled with [^{35}S]methionine 20 h after chloramphenicol treatment (see Materials and Methods).

Table II: Kinetic Analysis of β-Cystathionase from *E. coli* Cells

substrate	K_m (mM)	V_{max} (IU/mg)
L-cystathionine [a]	0.04	249
L-cystine [a]	0.25	263
L-homolanthionine [b]	4.54	255
L-*meso*-lanthionine [b]	0.83	262
L-djenkolic acid [b]	0.36	198

[a] Enzyme specific activity 237 IU/mg (see Materials and Methods). [b] Enzyme specific activity 196 IU/mg (see Materials and Methods).

pH Optimum and Enzyme Stability. The enzyme showed a bell-shaped pH–rate profile with an optimum between pH 8.0 and 9.0. At physiologic pH (7.2) the enzyme exhibited 36% of its optimal activity. The enzyme activity was determined in 0.1 M Tris–phosphoric acid buffer at various pH values between 6 and 10. The extinction coefficient of the reaction product at 412 nm did not vary between these pH values.

The purified enzyme was very stable when stored at 4 °C following sterile filtration and did not lose activity up to 4 months. However, the enzyme lost activity when frozen or lyophilized. When stored with 50% glycerol at 4 and −20 °C, the enzyme retained full activity up to 4 months.

Substrate Specificity. The purified β-cystathionase obeys Michaelis–Menten kinetics with all the substrates examined except for L-cysteine. The K_m values for L-cystathionine and L-cystine calculated from the Lineweaver–Burk plots (Lineweaver & Burk, 1934) were 0.04 mM and 0.25 mM, respectively (Table II). Both of these K_m values are lower than those reported for the *Salmonella* enzyme, 0.22 mM and 0.80 mM, respectively (Guggenheim, 1971). The V_{max} values for L-cystathionine and L-cystine were 249 and 263 units/mg of protein, respectively. L-Djenkolic acid, L-homolanthionine,

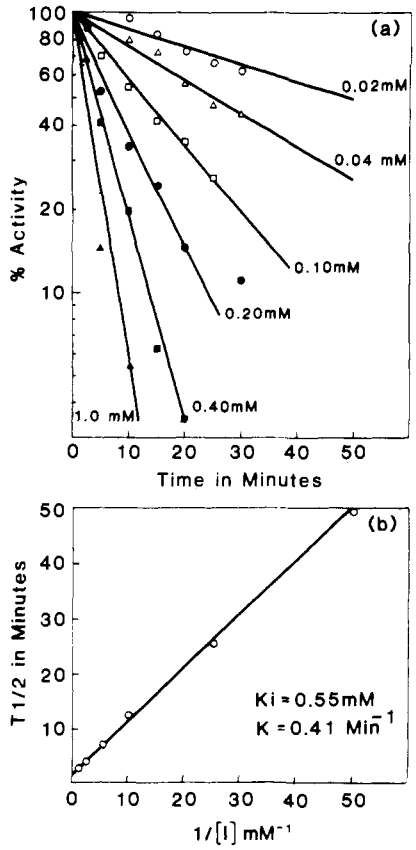

FIGURE 2: (a) Kinetics of inactivation of purified *E. coli* β-cystathionase by 3,3,3-trifluoroalanine. The enzyme (0.18 mg/mL) was incubated with various concentrations of the inhibitor in 0.1 M PBS buffer, pH 7.0, containing 1 mM EDTA at room temperature. Aliquots were withdrawn at various times and assayed for residual β-cystathionase activity by the aryl disulfide procedure of Flavin & Slaughter (1971). (b) Half-times of inactivation vs. reciprocal of inhibitor concentration.

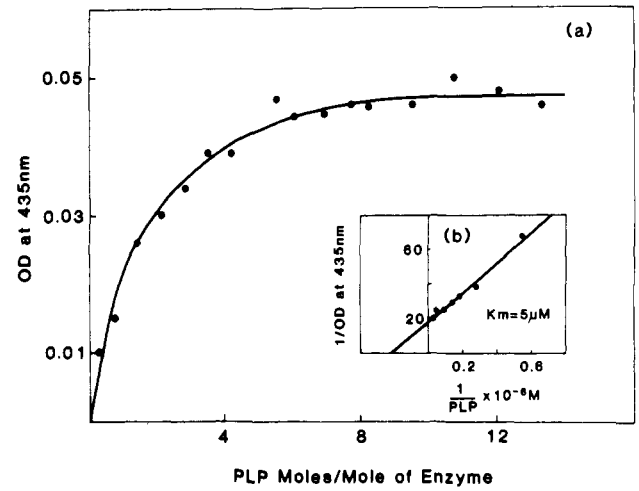

FIGURE 3: Spectrophotometric determination of amount of pyridoxal phosphate (PLP) binding to *E. coli* β-cystathionase. The apoenzyme was prepared by dialyzing the purified enzyme (0.7 mg/mL, protein determination on the basis of amino acid analysis data) against two batches of 250 mL of 0.05 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA, 0.1 mM DTE, and 50 mM cysteine to remove bound pyridoxal phosphate. The apoenzyme thus formed was further dialyzed against two batches of 1 L of 0.05 M sodium phosphate buffer, pH 7.0, containing 1 mM EDTA and 0.1 mM DTE to remove traces of cysteine. To the apoenzyme were then added aliquots of a concentrated solution of pyridoxal phosphate, and the increase in the absorbance at 435 nm was recorded on a Cary spectrophotometer. An incubation period of 30 min at room temperature was required to complete the reaction. (a) Typical saturation curve of apoenzyme with pyridoxal phosphate. (b) Double-reciprocal plot of absorbance vs. pyridoxal phosphate concentration.

and L-*meso*-lanthionine also served as substrates with K_m values of 0.4 mM, 5.0 mM, and 0.8 mM, respectively. L-Cysteine served as a poor substrate with a maximum velocity only 1% of the value with L-cystathionine, using the coupled enzyme assay system (Guggenheim, 1971) in the presence of 10 mM DTE. This substrate also showed excess substrate inhibition, which prevented a complete characterization. The *S. typhimurium* enzyme shows 7.5% of the cystathionine rate for this substrate (Guggenheim, 1971).

Enzyme Inhibition. The effect of various inhibitors on the activity of β-cystathionase was investigated. Adenosine (1 mM), *S*-adenosylmethionine iodide (1 mM), *S*-adenosylhomocysteine (1 mM), allylglycine (16 mM), diaminopimelic acid (16 mM), 2,7-diaminooct-4-ynedioic acid (16 mM), and propargylglycine (1 mM) did not show any significant inhibition of the enzyme activity. β-Cyanoalanine inhibited only about 43% of the enzyme activity at 16 mM concentration. L-Cysteine inhibited β-cystathionase competitively with L-cystathionine serving as substrate in the presence of 10 mM DTE. The inhibition constant (K_i) for L-cysteine was found to be 0.4 mM. This value is similar to the K_m value of L-cysteine (0.29 mM) with the *Salmonella* enzyme (Guggenheim, 1971).

Silverman & Abeles (1976) showed that 3,3,3-trifluoroalanine binds covalently with β- and γ-cystathionase and inactivates the enzyme irreversibly. This analogue is a potent inhibitor of the *E. coli* β-cystathionase as well. Figure 2a shows the time-dependent inhibition of β-cystathionase with various concentrations of 3,3,3-trifluoroalanine. A binding constant (K_i) of 0.55 mM and a saturating inactivation rate constant of 0.41 min^{-1} ($t_{1/2}$ = 1.7 min) (Figure 2b) were observed when half-times ($t_{1/2}$) of the enzyme activity were replotted vs. reciprocal of the inhibitor concentration.

Stoichiometry of Pyridoxal Phosphate Binding. The holoenzyme of β-cystathionase possesses an absorption maximum at 435 nm characteristic of many pyridoxal phosphate dependent enzymes (Wu & Morris, 1973). Titrations of apo-β-cystathionase with pyridoxal phosphate were carried out spectrophotometrically at 435 nm. Figure 3 shows the typical saturation curve (a) and the double-reciprocal plot of absorbance vs. pyridoxal phosphate concentration (b). A plot of the log of the absorption vs. moles of PLP added per mole of the apoenzyme indicated 5.7 mol of pyridoxal phosphate bound/mol of enzyme at saturation. These data together with the subunit molecular weight of 45 000 and aggregate size of 280 000 or six subunits per mol of enzyme suggest the binding of 1 mol of pyridoxal phosphate/subunit. A K_m of 0.005 mM for pyridoxal phosphate was calculated from the double-reciprocal plot of absorbance vs. concentration. The apo-β-cystathionase showed only 2.3% enzyme activity whereas the reconstituted holoenzyme exhibited 94.7% enzyme activity after complete saturation with pyridoxal phosphate following a 30-min incubation period. A molar extinction coefficient of 3680 M^{-1} cm^{-1} at 435 nm was calculated for pyridoxal phosphate binding from these studies.

Amino Acid Composition. The total amino acid composition of β-cystathionase is presented in Table III. Cysteine was the least frequent amino acid with 36 ± 1 residues per mole. Free SH titrations of the enzyme with DTNB demonstrated a total of 36.8 free SH groups/mol of protein, indicating that none of the cysteine residues are in a disulfide form. The rate of the titration with DTNB reagent indicated that most of these SH residues are not exposed to the surface since it required 4 h for complete reaction.

Table III: Amino Acid Composition of β-Cystathionase from *E. coli* Cells

residue	mol/mol of enzyme	mol/ subunit
Ala	284 ± 23	47
Arg	115 ± 9	19
Asx	246 ± 11	41
Cys[a]	36 ± 1	6
Glx	253 ± 19	42
Gly	214 ± 23	36
His	85 ± 18	14
Ile	154 ± 14	26
Leu	266 ± 24	44
Lys	120 ± 8	20
Met	36 ± 3	6
Phe	90 ± 7	15
Pro	82 ± 10	14
Ser	162 ± 3	27
Thr	114 ± 4	19
Trp[b]	54	9
Tyr	54 ± 4	9
Val	186 ± 10	31
integer M_r	279 594	46 561

[a] Determined as (carboxymethyl)cysteine. The carboxymethylation of the enzyme was achieved as described by Hirs (1967). [b] Determined spectrophotometrically as described by Edelhoch (1967).

Discussion

The value in obtaining recombinant DNA produced strains that carry the structural gene for β-cystathionase on a separately replicating plasmid is documented by the 100-fold overproduction of the enzyme over wild-type levels and 7-fold overproduction over constitutive levels. Similarly, these levels are 13-fold higher than those of extracts from *S. typhimurium* met-A-15, which was 7-fold higher than wild-type *Salmonella* (Guggenheim, 1971) and 6000-fold higher than spinach levels (Giovannelli & Mudd, 1971). The rapid three-step purification procedure described produces β-cystathionase of specific activity 27-fold higher than that of the *Salmonella* enzyme and 2000-fold higher than that of the spinach enzyme. The extent to which these differences reflect degrees of enzyme purity or kinetic properties cannot be judged due to the limited characterization of the other enzymes. The purity of the *E. coli* enzyme appears to be greater than 90% as judged by scans of stained acrylamide gel patterns and the agreement between the number of PLP bound (5.7) and subunits (280000/45000 = 6.2) per mole of enzyme and the number of cysteine residues determined by amino acid analysis (36) and by DTNB titration (36.8).

The broad substrate specificity of the *E. coli* enzyme is similar to those of the *Salmonella* and spinach enzyme and rat liver γ-cystathionase. The cystine turnover is much greater for the *E. coli* enzyme (V_{max} = 263 IU/mg) than for the rat liver γ-cystathionase (V_{max} = 0.29 IU/mg) (Uren et al., 1978). The cysteine inhibition constant is similar between the β and γ enzyme although the β enzyme appears to turn over cysteine at a slow rate (1% for the *E. coli* enzyme and 7.5% for the *Salmonella* enzyme). The γ enzyme is strongly inhibited by both propargylglycine and β-cyanoalanine (Uren et al., 1978) while the *E. coli* β enzyme is not inhibited by these compounds. The spinach β-cystathionase is also inhibited by β-cyanoalanine (Giovannelli & Mudd, 1971). 3,3,3-Trifluoroalanine inactivates both the γ- and β-cystathionase enzymes (Silverman & Abeles, 1976).

Previous reports from this laboratory have documented the ability of cysteine- and cystine-degrading enzymes to inhibit the growth of cysteine-dependent neoplasms in culture and the pharmacologic and kinetic limitations of both cysteine desulfhydrase and γ-cystathionase for in vivo therapy (Uren & Lazarus, 1979; Uren et al., 1978). As a continuation of these efforts, β-cystathionase offers the following advantages over the previously examined enzymes. (1) The β enzyme has a 1000-fold higher specific activity for cystine decomposition over the γ enzyme. This low cystine turnover for the γ-cystathionase was the primary limitation of the enzyme for in vivo therapy. (2) The β enzyme is not inhibited by propargylglycine such that simultaneous therapy with the inhibitor of cysteine biosynthesis and the cystine-degrading enzyme could be performed. (3) The product of cystathionine cleavage with the β enzyme is homocysteine, which will not support malignant cell growth in the absence of cystine unlike the cysteine produced by γ-enzyme cleavage. (4) The β enzyme shows 36% of its maximal activity at pH 7.2 while cysteine desulfhydrase shows less than 5% of the maximal rate at pH 7 (Kredich et al., 1973). (5) The β enzyme shows hyperbolic substrate–velocity kinetics unlike the sigmoidal kinetics exhibited by cysteine desulfhydrase. Consequently, reaction velocities drop linearly rather than logarithmically as the substrate becomes depleted. (6) All three enzymes have K_m values of 0.1–0.3 mM, close to the plasma cysteine–cystine concentration.

Unfortunately, β-cystathionase like cysteine desulfhydrase is cleared rapidly from the plasma of mice (unpublished observations), which has precluded therapeutic trials. Current efforts are directed at chemically modifying the enzyme by the attachment of polymers of DL-alanine to improve its pharmacologic properties. Such chemical modification procedures have greatly improved the therapeutic, immunological, and clearance properties of both the *E. coli* and *Erwinia carotovora* L-asparaginases (Uren & Ragin, 1979).

Acknowledgments

We thank Dr. Michael Berman for his gift of the streptomycin-resistant GUC41 strain and for valuable discussions throughout the course of the molecular genetic studies.

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Preparation and Characterization of Fluorescent 50S Ribosomes. Specific Labeling of Ribosomal Proteins L7/L12 and L10 of *Escherichia coli*†

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ABSTRACT: So that the topographic and dynamic properties of the L7/L12–L10 complex in the 50S ribosome of *Escherichia coli* could be studied, methods and reagents were developed in order to introduce fluorescent groups at specific positions of these proteins. In the case of L7/L12, this was done by attaching an aldehyde group to Lys-51 of the protein by using 4-(4-formylphenoxy)butyrimidate or by converting the amino terminus of L12 into an aldehyde group by periodate oxidation. Subsequent reaction of the aldehyde groups with newly developed hydrazine derivatives of fluorescein and coumarin resulted in specifically labeled L7/L12 derivatives. L10 was modified at the single cysteine residue with *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide. The fluorescent proteins L10 and L7/L12 could be reconstituted into 50S ribosomes. The resulting specifically labeled 50S ribosomes show 25–100% activity in elongation factor G dependent GTPase as well as in polyphenylalanine synthesis. The fluorescent properties of the labeled 50S ribosomes show that these fluorescent derivatives are suitable for energy transfer studies.

A precise understanding of the mechanism of function of the ribosome requires detailed structural information on the components and the distances to each other. For the latter, several methods are used in order to achieve this goal [for a recent review, see Chamblis et al. (1980)], for example, neutron scattering (Moore, 1980) and fluorescence energy transfer of randomly labeled proteins (Cantor et al., 1974). In both cases, a distance between the centers of mass of two specified proteins is obtained, but the conversion of these data into the actual three-dimensional picture requires the knowledge of the shape of the protein, something which is hard to obtain. In the case of the fluorescence energy transfer, the assumption of a random labeling also is questionable. Distances between specifically labeled sites lead to more insight into the topography of the ribosome. This approach has been performed initially with specifically labeled fluorescent tRNAs and erythromycin bound to the ribosome (Langlois et al., 1976; Fairclough & Cantor, 1979), while also 30S (Kang et al., 1979) and 50S ribosomes (Lee et al., 1981) have been labeled with some specificity to respectively the cysteine of S18 and Lys-120 of L7/L12.

In this paper, we report the preparation of specifically labeled L7/L12 and L10. In the case of L7/L12 (L7 is the N-acetylated form of L12), a new technique was developed which enabled us to prepare L7, specifically labeled at Lys-51, and L12, labeled at the N terminus. The properties of these modified proteins with respect to binding to ribosomal core particles and in the functional reconstitution of core particles are described.

In the following paper (Zantema et al., 1982), some of these proteins are used for distance measurements between L10 and L7/L12 on the 50S ribosomal particle.

Experimental Procedures

The following buffers were used: TMA buffer, 20 mM Tris-HCl, pH 7.6, 10 mM $Mg(OAc)_2$, 10 mM NH_4Cl, and 6 mM 2-mercaptoethanol; GTPase buffer, 20 mM Tris-HCl, pH 7.6, 10 mM $Mg(OAc)_2$, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol; CM buffer, 6 M urea, 25 mM acetic acid–piperidine, pH 5.2, 1 mM ethylenediaminetetraacetic acid (EDTA),[1] and 1 mM DTE.

NaB^3H_4, [^{14}C]formaldehyde, [γ-^{32}P]GTP, and [^{3}H]-phenylalanine were obtained from Amersham. Urea was deionized before use. Protein concentrations were determined according to Lowry et al. (1951) with insulin as standard. EF-G, EF-Tu, and EF-Ts were isolated according to Arai et al. (1972).

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[1] Abbreviations: EF-G, elongation factor G; EF-Tu, elongation factor Tu; EF-Ts, elongation factor Ts; TLC, thin-layer chromatography; FPB-L7, 4-(4-formylphenoxy)butylamidine-L7; FAPB-L7, 4-(6-formyl-3-azidophenoxy)butylamidine-L7; DACM-L10, adduct of *N*-[7-(dimethylamino)-4-methylcoumarinyl]maleimide with L10; EDTA, ethylenediaminetetraacetic acid; DTE, 1,4-dithioerythritol; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/82/0421-3069$01.25/0