Kinetics and Inhibition of Recombinant Human Cystathionine γ-Lyase

TOWARD THE RATIONAL CONTROL OF TRANSSULFURATION*

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The gene encoding human cystathionine γ-lyase was cloned from total cellular Hep G2 RNA. Fusion to a T7 promoter allowed expression in Escherichia coli, representing the first mammalian cystathionine γ-lyase overproduced in a bacterial system. About 90% of the heterologous gene product was insoluble, and renaturation experiments from purified inclusion bodies met with limited success. About 5 mg/liter culture of human cystathionine γ-lyase could also be extracted from the soluble lysate fraction, employing a three-step native procedure. While the enzyme showed high γ-lyase activity toward L-cystathionine (K_m = 0.5 mM, V_max = 2.5 units/mg) with an optimum pH of 8.2, no residual cystathionine β-lyase behavior and only marginal reactivity toward L-cystine and L-cysteine were detected. Inhibition studies were performed with the mechanism-based inactivators propargylglycine, trifluoroalanine, and aminoethoxyvinylglycine. Propargylglycine inactivated human cystathionine γ-lyase much more strongly than trifluoroalanine, in agreement with the enzyme’s preference for C-γ-S bonds. Aminoethoxyvinylglycine showed slow and tight binding characteristics with a K_i of 10.5 μM, comparable with its effect on cystathionine β-lyase. The results have important implications for the design of specific inhibitors for transsulfuration components.

Transsulfuration and reverse transsulfuration constitute part of the metabolic interconversion of the sulfur-containing amino acids cysteine and methionine (Fig. 1). The forward pathway, the transformation of cysteine into homocysteine via the intermediate L-cystathionine is catalyzed by the sequential action of the enzymes cystathionine β-lyase (CBL) and cystathionine γ-synthase (CGS) and has been identified in bacteria, fungi, and plants. Conversely, reverse transsulfuration, catalyzed by the enzymes cystathionine β-synthase and cystathionine γ-lyase (CGL), is known only in fungi and mammals (1, 2). Actinomycetes species present a notable exception to this rule (1). The four enzymatic transsulfuration components are all pyridoxal 5′-phosphate (PLP)-dependent enzymes, but they pertain to different structural groups; CBL, CGS, and CGL show extensive sequence homology and are members of the PLP γ-family (Ref. 3; Fig. 2), while cystathionine β-synthase is unrelated and belongs to the β-family.

The high resolution crystal structures of Escherichia coli CBL (4) and CGS (5), together with crystallographic (6) and kinetic investigations (6–9) on inhibitors, allowed the suggestion and evaluation of reaction mechanisms (4, 10). Both CBL and CGS are homotetramers composed of ~40–45-kDa subunits and carry one PLP cofactor per monomer covalently bound via a Schiff base to an active site lysine. A similar situation has been found for CGL (1, 11, 12). In the present paper, we extend our structure-function analyses to human CGL (EC 4.4.1.1; unless otherwise stated, “CGL” refers to human cystathionine γ-lyase). CGL catalyzes the second step in the reverse transsulfuration pathway, i.e. the cleavage of the L-cystathionine C-γ-S bond, yielding L-cysteine, α-ketobutyrate, and ammonia (Fig. 1). In humans, the enzyme is linked to cystathioninuria, cystinosis, and (although less frequently than cystathionine β-synthase (13)) homocystinuria (14). These metabolic disorders potentially result in mental or physical impairments. In addition, malignant lymphocytic cells show markedly reduced levels of CGL and do not grow in media devoid of L-cystine (14–16). Therefore, observations of L-cyst(e)ine conversion by CGL from various species (1, 11, 14, 17, 18) suggested the possibility of exploiting the enzymatic activity for cyst(e)ine depletion in blood (14). Alternatively, intracellular inhibition of the enzyme could block a metabolic route toward L-cyst(e)ine (14). Furthermore, since human hepatic CGL activity levels are markedly reduced compared with those in rat liver, CGL could be a rate-limiting factor in human liver glutathione synthesis (through the limited availability of cysteine), which could be ultimately responsible for a susceptibility to alcoholic liver damage (Ref. 19 and references therein).

Despite the central metabolic role of CGL and its link to a broad pathological spectrum, until now detailed mechanistic or structural data on the human enzyme are lacking. Such studies have been severely hampered by the small overall yields of native isolations (14). While nonrecombinant CGL purifications from several species have been described (neurospora (20), streptomycyes (1), and rat (14, 17)), the yeast enzyme so far is the only one that has been heterologously expressed in E. coli (11). The reported CGL preparations have not been exhaustively characterized, particularly with respect to substrate specificities and the enzymes’ responses toward inhibitors. Inhibition of the transsulfuration enzymes is of interest because the different enzymatic spectra displayed by different organisms (Fig. 1) could be exploited for the development of new antibiotics and herbicides. Inhibition studies were so far focused on CGS and CBL, the enzymes of methionine biosynthe-
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Preparation of Total Cellular RNA, Cloning, and Sequencing—Total RNA from Hep G2 liver cells (ATCC-No. HB-8065) was prepared according to Chirgwin et al. (25). Briefly, after quantitative removal of the culture supernatant, 300 cm² of confluent cells were lysed with 4 ml of denaturing solution (4 mM guanidinium thiocyanate, 25 mM sodium citrate, 0.5% (w/v) Na-lauroylsarcosine, 0.1 M 2-mercaptoethanol). The DNA was sheared by passing the lysate 10 times through a pipette. After the addition of 0.1 g of acryl per ml of lysate, the solution was layered over a cushion of 9.5 M CsCl in a SW28 tube (Beckman) and centrifuged at 22 °C overnight at 113,000 × g. The supernatant was removed, and the RNA pellet was immersed overnight at 4 °C in 3 ml of 50 mM EDTA, 0.5% (w/v) Na-lauroylsarcosine, and 5% (v/v) 2-mercaptoethanol. After the addition of 0.1 volume of 3 M sodium acetate, pH 5.2, the RNA was precipitated with 2.5 volumes of ethanol at −70 °C for 30 min. The total RNA was centrifuged at 15,000 × g for 15 min, dried, resuspended in water, and stored at −70 °C. The presence of RNA in the required size range was confirmed by urea polyacrylamide gels with 16 and 23 S ribosomal RNA as standards. 100 pmol of an oligo(dT)15 primer served for first strand cDNA synthesis with 25 μl of total Hep G2 RNA in a 50-μl reaction. Other components and reaction conditions were as suggested by the RT supplier. Second strand cDNA synthesis and amplification of the product were performed via polymerase chain reaction (26, 27) in 50-μl reaction volumes with 5 μl of the column-purified RT reaction mix as the template (30 polymerase chain reaction cycles: 30 s at 95 °C, 60 s at 55 °C, 12 min at 68 °C) and 100 pmol of 5′-overhang primers (forward primer, 5′-CGATGGGTACCATGTGACAGAAAAGAGCGCTTCCTCACAAGG-3′; reverse primer, GGGTACCC-TGAGTTACTACTGTGAAATTTCCACTTTGGAAGGCGTGTCCGTCG-3′; sequences corresponding to the human CGL gene are italicized). The primers contained restriction enzyme recognition sites (forward primer Ndel; reverse primer Xhol; underlined) for subsequent cloning into a pET228(+). (Novagen, Madison, WI) expression vector. The amplified cDNA and the vector preparation were digested overnight with Ndel/Xhol at 37 °C and purified via a 1% agarose gel. Ligation occurred overnight at 16 °C. Individual colonies were obtained from E. coli XL1-Blue cells electroporated with the ligation mixture, grown on LB plates supplemented with 100 μg/ml ampicillin (LB-Ampl100), and propagated in liquid LB-Ampl100 medium for plasmid preparations. Potentially positive clones were identified through restriction digests and verified by automated Sanger dideoxynucleotide sequencing. In a similar fashion, the CGL gene was cloned behind a Ptac promoter in the pCYB1 expression vector (New England Biolabs).

Heterologous Expression in E. coli—40 μl of electrocompetent BL21(DE3) E. coli cells were electroporated with 0.5 μg of plasmid DNA at 18,000 V/cm (Electroporator 1000, Stratagene). Transformants were rescued in 1 ml of LB for 1 h at 37 °C and used to inoculate an overnight 100-ml LB-Ampl100 starter culture. 3 ml of the starter culture were propagated in 3-liter Erlenmeyer flasks with 650 ml of LB-Amp200. After shaking at room temperature to an A600 of ~0.7, 1 mM isopropyl-β-D-thiogalactopyranoside was added, and the cultures were maintained at room temperature for another 12 h. Cells were harvested by centrifugation in a Beckman JS4.2 rotor at 4200 rpm for 30 min, resuspended in 7 ml/liter original culture of resuspension buffer (50 mM KP, buffer, pH 8.0, 0.5 mM PLP, and 2 mM EDTA), and stored at −70 °C until further processing. 15% polyacrylamide SDS gels showed a strong expression band at about 45 kDa (Fig. 3b), which was roughly distributed in a 10:90 ratio between soluble and insoluble fractions, respectively, as estimated by the Coomassie stain.

purification under native conditions—Cells from the 6-liter overnight culture were thawed and, after the addition of 0.1 mm phenylmethylsulfonyl fluoride, incubated with 0.3 μg/ml hen egg white lysozyme for 30 min at room temperature. Cell lysis and DNA shearing were completed by 15-min sonication on ice using the 50% pulsed maximum output of a Branson (Danbury, CT) sonifier equipped with a macrotip. All subsequent steps were performed with the protein on ice or at 4 °C. The lysate was cleared for 45 min at 20,000 rpm in a JA25.50 rotor. The supernatant was applied to a 300-ml DEAE-Sepharose FF column equilibrated with 20 mM Tris (pH 7.6), buffer, pH 8.0, 0.1 mM PLP, 2 mM EDTA. A linear 1-10 gradient run at 8 ml/min to buffer A plus 0.5 mM ammonium sulfate eluted CGL at about 0.1 μl salt. Fractions containing CGL were identified via enzymatic assays (see below) and SDS-polyacrylamide gels. The DEAE pool was adjusted to 1.0 mM ammonium sulfate, loaded on a 100-ml phenyl-Sepharose HP column, equilibrated with buffer A plus 1.0 mM ammonium...
Preparation of Inclusion Bodies and Renaturation—The CGL-expressing cells were harvested and lysed as before. The pellet from the cell lysis was resuspended in 50 mM sodium phosphate, 300 mM NaCl, pH 7.8, 0.5% N,N-dimethyldecylamine-N-oxide, sonicated (Branson, macrotip, 80% pulsed, 80% output for 15 min), and again centrifuged. Washing was repeated once with N,N-dimethyldecylamine-N-oxide and twice without detergent. 150 mg of purified inclusion bodies were dissolved in 10 ml of solubilization buffer (6 M guanidinium hydrochloride, 150 mM NaCl, 2 mM EDTA, 20 mM PLP, 5 mM 1,4-dithiothreitol, 100 mM Tris/HCl, pH 8.5), slowly dripped overnight at 4 °C into a 500-ml reservoir of renaturation buffer (150 mM NaCl, 2 mM EDTA, 20 mM PLP, 5 mM 1,4-dithiothreitol, 100 mM Tris/HCl, pH 8.5), and subsequently dialyzed against three changes of 4 liters of (a) 150 mM NaCl, 2 mM EDTA, 20 mM PLP, 100 mM Tris/HCl, pH 8.5, (b) 50 mM NaCl, 2 mM EDTA, 10 mM PLP, 100 mM Tris/HCl, pH 8.5, and (c) 50 mM NaCl, 2 mM EDTA, 10 mM PLP, 20 mM Tris/HCl, pH 8.5. The soluble supernatant was then passed over a 50-ml DEAE-Sepharose FF column at 4 ml/min with a 400-ml linear gradient from 50 mM NaCl, 2 mM EDTA, 10 mM PLP, 20 mM Tris/HCl, pH 8.5, to the same buffer containing 0.5 M ammonium sulfate. Analytical of the fractions was performed as before. Similar renaturation trials were attempted using renaturation buffers with 1.2 M L-arginine and direct dialysis against guanidinium-free buffers.

Protein Determination—Protein concentrations were measured by the method of Bradford (28) with a bovine serum albumin standard curve. For calculation of kinetic constants, CGL concentrations were determined by the 280-nm absorption using the calculated molar extinction coefficient $\varepsilon_{280} = 30,500 \text{ M}^{-1} \text{cm}^{-1}$ and a subunit molecular weight of 44,534.

Determination of the pH Optimum—Solutions of 0.8 mM L-cystathionine in 40 mM borate buffer at pH 7.8 and 9.4 were titrated against each other to build a pH profile. 985 $\mu$M of these buffers were mixed with 10 $\mu$M of 0.1 M DTNB in ethanol and 5 $\mu$M of enzyme (6 mg/ml). The linear portion of the reaction time course was monitored at 30 °C for 60 s through the development of a 412-nm absorption. Subsequent kinetic analyses were performed at the determined optimum pH of 8.2 for the native enzyme preparation.

Michaelis-Menten Kinetics—Detailed kinetic analyses were only performed with CGL purified under native conditions. Time courses for the CGL reaction were monitored at 30 °C for 1 min using the above direct colorimetric assay with DTNB. The 1-mM reactions contained 985 $\mu$M of 40 mM borate buffer, pH 8.2, adjusted to L-cystathionine concentrations between 0.03 and 3.0 mM, 5 $\mu$M of CGL (6 mg/ml), and 10 $\mu$M of 0.1 M DTNB in ethanol. After rapid mixing, the development of the 412-nm absorption was followed for 1 min. Kinetics were linear for this time and throughout this concentration range. Data were plotted in reciprocal form according to Eadie and Hofstee (29, 30) to extract values for $K_m$ and $V_{max}$. To evaluate the substrate specificity of CGL, identical experiments were performed in which L-cystathionine was replaced by L-
cystine or l-cysteine. L-Cysteine levels were also monitored in a discrete fashion as described below. L-Cysteine conversion was followed by the above DTNB assay and another continuous examination employing lactate dehydrogenase to detect α-ketoacids produced in the reaction via oxidation of NADH (20).

Monitoring of Cysteine Levels—L-Cysteine was quantitated according to Gaitonde (31). 80-μl samples were removed from CGL reactions (borate buffer with l-cystathionine and enzyme) and mixed with 30 μl of 20% trichloroacetic acid, and the precipitated protein was removed by centrifugation. 80 μl of the terminated reactions were mixed with equal amounts of acetic acid and ninhydrin reagent (250 mg of ninhydrin dissolved in 6 ml of acetic acid and 4 ml of hydrochloric acid), boiled for 10 min, and diluted with 1 ml of 95% ethanol. Development of the pink color was monitored at 560 nm. The amount of l-cysteine was calculated using the published molar extinction coefficient, ε560 = 2.6 × 10⁴ M⁻¹ cm⁻¹. A standard curve with known amounts of l-cysteine in reaction buffer, treated in the same way as the reactions, served to verify the linearity of the response in the relevant concentration range. References contained all buffer and reaction components with the exception of l-cysteine or l-cysteine-producing precursors. Under the given conditions, l-homocysteine, l-cystine, and l-methionine do not show a similar response (31).

Inhibition Kinetics—Inhibition of CGL by AVG was detected in 30-min time courses, monitored by the DTNB assay. Reaction buffers contained saturating amounts of l-cystathionine (2.5 mM) in 40 mM borate buffer, pH 8.2, and varying levels of inhibitor. The resulting progress curves were analyzed according to Cha (32, 33), Morrison (34), and Morrison and Walsh (35).

Inactivation of CGL by PG and F₄Ala was studied by reacting 2.5 mM l-cystathionine in 40 mM borate buffer, pH 8.2, with set amounts of CGL, preincubated for given times at 37 °C with varying amounts of the inhibitors. Reactions were monitored for 60 s in the continuous DTNB assay. From the activity remaining at various times after incubation with set amounts of F₄Ala, the half-times of inactivation (t½) were determined. A Kitz-Wilson analysis (Ref. 36; inverse inhibitor concentration) was fitted to extract the inhibition constant, Kᵢ, and the rate of inactivation, kᵢnact. To analyze the inactivation by PG, normalized activities, remaining after given times of incubation, were plotted against the relative concentrations of PG and enzyme active sites.

**RESULTS**

Cloning and Recombinant Expression of Human CGL—After 30 cycles of polymerase chain reaction and restriction digestion, a single product of the expected size (1218 base pairs) was visible on ethidium bromide-stained agarose gels (Fig. 3a). The cloned full-length CGL gene corresponded to one of the published sequences (19). Previously, similar cloning strategies have met with a second product with an internal 132-base pair deletion compared with the rat liver CGL gene (19). We could not detect such a heterogeneity in the present RT assays. CGL shows homology throughout its entire length not only to CGL from other species but also to CGS and CBL (Fig. 2), two other enzymes of the transsulfuration pathways, and from the crystal structures of E. coli CBL (4) and CGS (5), specific functions can be attributed to the domains and key residues of the protein and its relatives. The observed deletion in the shorter CGL mRNA species would correspond to a severe trimming of the PLP binding domain including several catalytically indispensable residues (Fig. 2). Presumably, an enzyme with the detected deletion would be inactive.

Expression of CGL in E. coli under control of a T7 promoter was, at various culture conditions, reproducibly accompanied by the formation of insoluble inclusion bodies. In order to enhance solubility by reducing the expression level, fermentation was performed at isopropyl-β-D-thiogalactopyranoside concentrations of 0.1–1 mM, at varying temperatures (18, 25, 37 °C), and with the gene under control of a Pₛₐₑ promoter in several E. coli strains (BL21(DE3), XL1-Blue, and DH5α). Neither alone nor in combination did these factors significantly influence the degree of solubility of CGL. Induction of the Pₛₐₑ construct produced a soluble ~50-kDa band on SDS gels, which was characterized as E. coli tryptophanase (not shown), while no overexpression corresponding to CGL was observed. We do not have an explanation for our failure to express CGL with the latter promoter system. All further results therefore refer to enzyme produced with the T7-promoter system.

**Purification and Biophysical Characterization**—In order to have at hand large amounts of native CGL for detailed mechanistic and structural studies, we developed a swift purification procedure for the soluble fraction of the protein (Table I). The product was over 95% pure as judged from Coomassie-stained SDS-polyacrylamide gels (Fig. 3b). The integrity of the purified samples was attested by their strong activity toward the in vivo CGL substrate L-cystathionine (see below) and by UV-visible spectra showing the expected maxima at 280 nm (protein) and 427 nm (protein-bound PLP) (Fig. 4). Considering the similar protein sizes of the rat and human enzymes and the abundance of tryptophan residues (rat liver CGL: 2; human CGL: 3), the 280 nm/427 nm ratio of human CGL (7.5) is comparable with the value of 5 seen with rat liver CGL (17). The absorption spectra of the enzyme were virtually indistinguishable in bovine, phosphate, or Tris buffers. An additional minor maximum was observed at 494 nm (Fig. 4). When CGL apoenzyme was prepared by extensive dialysis against buffer containing l-alanine, both long wavelength absorption maxima disappeared, suggesting that they are both PLP-related and not due to contaminants (Fig. 4). This conclusion is in agreement with the unique band on SDS gels and the unequivocal N-terminal sequence of the product. However, when PLP was reincorporated into the CGL apoenzyme by dialysis and gel filtration, the 494-nm absorption band did not reappear.

Elution times of the purification product on an analytical Superose-12 size exclusion column were identical within experimental error to those of CGL from E. coli (monomer molecular mass ~40 kDa; not shown) which has been proven to exist in tetrameric form (10, 37). This shows that, analogous to the latter enzyme, CGL forms a homotetramer under native conditions, consistent with previous reports from other sources (1, 11, 12).

Because of the large amount of CGL deposited in insoluble form, we also attempted denaturing purification with subsequent in vitro folding of the protein. Fast dilution into guanidinium-free buffers yielded a soluble protein, verified as CGL by N-terminal sequencing. However, instead of the strong absorption at 427/494 nm due to the PLP cofactor, UV-visible spectra of the backfolded fraction showed a smaller absorption at 326 nm. The pH optimum was observed at pH 8.7, almost half a pH unit higher than that of the native protein (see below) and was not as clearly defined (Fig. 5a), while the catalytic

| Purification step                             | Volume | Protein concentration | Protein mass | Activity | Specific activity | Recovery | Enrichment factor |
|----------------------------------------------|--------|-----------------------|--------------|----------|------------------|----------|------------------|
| Soluble cell extract                         | 80     | 13.4                  | 1072         | 150      | 0.14              | 100      | 1.0              |
| DEAE anion exchange chromatography           | 105    | 2.6                   | 276          | 116      | 0.42              | 77       | 3.0              |
| Phenyl-Sepharose chromatography              | 70     | 0.8                   | 56           | 95       | 1.7               | 63       | 12.0             |
| Size exclusion chromatography                | 45     | 0.7                   | 31.5         | 78.9     | 2.5               | 53       | 18.0             |

**TABLE I**

**Purification of human CGL overexpressed in E. coli**
parallel lines obtained in double reciprocal plots (Fig. 5b; see below) for the native and the folded preparations suggest that only a minor fraction of the renatured samples was correctly folded. We refrained from additional purification steps because of the small expected yields. All of the following analyses refer to the native purified CGL.

**Kinetic Analyses**—The pH optimum of the CGL reaction with L-cystathionine was determined in borate buffer and located at pH 8.2 (Fig. 5a). Contrary to previous reports for rat CGL (12), we have not found pronounced differences when other buffers (Tris, phosphate) were used, consistent with the spectral results. All subsequent kinetic data were recorded in borate buffer, pH 8.2. $K_m$ and $V_{max}$ values for human CGL with respect to the in vivo substrate L-cystathionine were extracted from double reciprocal plots (Fig. 5b; $K_m = 0.5$ mM, $V_{max} = 2.5$ units/mg), and $k_{cat}$ was calculated to 1.9 s$^{-1}$. Interestingly, despite their highly conserved features (Fig. 2) and similar catalytic efficiencies with respect to the in vivo substrate L-cystathionine, the CGL enzymes from different species exhibit order-of-magnitude differences in their $K_m$ and $V_{max}$ values (Table II).

CGL from yeast (11) and rat liver (14) show an appreciable CBL-like activity, i.e., they are able to cleave both the C-$\gamma$-S and C-$\beta$-S bonds of L-cystathionine. In contrast, with human CGL and the substrate L-cystathionine, the appearance of total product sulfhydryl groups (detected in the DTNB assay) perfectly matched the developing L-cysteine levels (detected by Gaitonde’s method (31); Fig. 6a), showing that the substrate was almost exclusively split at the C-$\gamma$-S bond. L-Cysteine was degraded at least 2 orders of magnitude slower than L-cystathionine at respective concentrations of 0.55 mM (Fig. 6b). Similarly, L-cysteine degradation was not detectable through product $\alpha$-ketoacids (lactate dehydrogenase assay), while the DTNB assay showed a very small response (Fig. 6b). Both reactions, degradation of L-cystine and L-cysteine, involve the splitting of C-$\beta$-S bonds. Therefore, we observe a high reaction specificity of human CGL toward C-$\gamma$-S bonds.

**Slow Binding Inhibition by AVG**—Reaction curves obtained with CGL in the presence of both AVG and saturating amounts of L-cystathionine displayed a slow decrease of the reaction rate. They finally reached a steady state velocity, which was dependent on the concentration of the inhibitor. In order to exclude the possibility that decomposition or modification of AVG by CGL caused the slow arrival at the steady state levels, CGL was preincubated with AVG before the addition of the substrate. Subsequent activity assays indicated the same steady state velocities. Thus, AVG shows slow binding inhibition (38) of CGL.

Three simple mechanisms could account for the observed slow binding inhibition (Refs. 34 and 35; Table III): (I) slow binding of inhibitor, I, to the enzyme, $E$; (II) rapid binding of I to $E$ followed by a slow isomerization step to $EI^*$; and (III) slow isomerization of $E$ into a state $E^{**}$ capable of rapidly binding I. In order to gain insight into the mechanism in the present case, the slow coupling of CGL and AVG was further characterized by recording reaction progress curves in the presence of varying amounts of inhibitor over prolonged times. These progress curves showed no dependence of the initial reaction velocity, $v_o$, on the inhibitor concentration. The time traces were fitted to Equation 1 (Table III) to extract apparent reaction rate constants, $k_{obs}$. A plot of the obtained $k_{obs}$ values against the inhibitor concentration (Fig. 7a) resulted in a straight line without any indication of saturation kinetics. These observations suggest the direct, slow establishment of a tight, inactive $EI$ complex without a rapid pre-equilibrium (mechanism I, Table III). For this mechanism, $k_{obs}$ is composed as shown in
### Table II

| CGL source      | Substrate | $K_m$ | $V_{max}$ | $V_{max}/K_m$ | Reference |
|-----------------|-----------|-------|-----------|---------------|-----------|
| Human liver     | L-cystathionine | 0.5   | 2.5       | 5.0           | This work |
| Rat liver       | L-cystathionine | 3.0   | 12.3      | 4.1           | 17        |
|                 | L-cystathionine | 3.5   | 4.6       | 1.3           | 14        |
|                 | L-cystathionine | 0.07  | 0.29      | 4.1           | 14        |
| Yeast           | L-cystathionine | 0.25  | 0.71      | 2.8           | 11        |
| Streptomyces    | L-cystathionine | 0.20  | 1.37      | 6.9           | 1         |
|                 | L-cystine     | 0.91  | 4.73      | 5.3           | 1         |
| Neurospora *    | L-cystine     | 0.03  |           |               | 20        |

*Sequence data not available.

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**Equation 2** (Table III), in which $k_2$ represents the dissociation rate. Fitting of $k_{obs}$ to Equation 2 yields a rate constant, $k_2$, of $3.4 \times 10^{-3} \text{ s}^{-1}$ and a value of 10.5 $\mu$M for the dissociation constant, $K_1$. For $k_1$, a value of 324 $\text{M}^{-1} \text{s}^{-1}$ was calculated using the equation $K_1 = k_2/k_1$.

The above analysis assumes the simplest mechanism for slow binding inhibitions, *i.e.* slow formation of an EI complex. An indication that mechanism I does not perfectly explain the present data was obtained by replottting $1/(k_{obs} - k_2)$ versus $1/[[I]]$, which did not result in the expected straight line but showed hyperbolic behavior. The latter observation favors the fast formation of a weak EI complex that slowly isomerizes to a tight EI* complex (mechanism II, Table III). This mechanism is indeed found for most slow binding inhibition reactions (34, 35, 38). The seeming discrepancy with the results favoring mechanism I can be explained, assuming that in the present case $K_i$ (describing the initial EI complex formation) is much greater than the overall dissociation constant $K_{i*}$ (describing the entire reaction to EI*). If, as in the present analysis, the inhibitor concentration is varied in the range of $K_{i*}$ ($\mu$M), one works with inhibitor concentrations much lower than $K_i$ (in the millimolar range). Under these conditions, the rate equation for $k_{obs}$ for mechanism II simplifies (35) from Equation 3 (Table III), with $k_2$ as the dissociation rate of EI*, to Equation 4 (Table III), which is indistinguishable from Equation 2. It is therefore correct to analyze the data in the present case based on the equation for mechanism I.

The binding of AVG was also monitored via UV-visible spectra, showing a decrease of the pyridoxaldimine absorbance at 427 nm and the appearance of a new chromophore at 330 nm (Fig. 4). In order to elucidate whether the observed inhibition by AVG was reversible, AVG-inhibited enzyme was dialyzed extensively against AVG-free buffer. Almost full enzymatic activity (more than 90% of the initial activity) could be restored. The regain of the enzymatic activity was accompanied by the reappearance of the 427-nm pyridoxaldimine absorbance and the disappearance of the 330-nm absorption maximum for the AVG-inhibited enzyme. These results suggest that AVG acts on CGL by slow and tight, but reversible, binding to the active site PLP cofactor, thereby changing the absorption characteristics of the cofactor.

**Suicide Inhibition by PG and F3Ala**—Contrary to AVG, PG inactivated the enzyme faster than the manual mixing time for the samples. The irreversible inactivation of CGL by PG has been previously studied using the rat enzyme (39), and the following mechanism was proposed. Absorption of a $\beta$-proton leads to an allene that is capable of Michael addition to an enzyme active site nucleophile (Fig. 8c). The very fast inactivation of CGL by PG was therefore not surprising, since the covalent coupling takes place at the $\gamma$-carbon, *i.e.* the site of attack of CGL in the physiological substrate L-cystathionine. Because of the rapidity of the inactivation, we determined the activity levels remaining after a 10-min incubation of CGL with varying amounts of inhibitor. When the data are represented relative to the concentration of active sites (Fig. 7b), it can be seen that 1 eq of PG inactivates 1 eq of active sites. This stoichiometry is in contrast to the results with the rat enzyme, where two active sites were inactivated by 1 PG eq (39). Still, PG shows extremely strong inhibition of CGL, since almost every PG molecule leads to the inactivation of a CGL active site (Fig. 7b).

In contrast to PG, F3Ala has to react at its $\beta$-carbon, explaining its much slower inhibition of CGL. Due to the slower reaction, we could conveniently study inactivation by F3Ala with a manual assay. CGL was incubated with F3Ala, and aliquots were removed at several time points to determine residual CGL activity. A biphasic behavior was observed for the activity...
plotted against the incubation time; a fast initial inactivation was followed by a slower one for longer incubation times. This behavior is often observed for irreversible inactivation processes and is due to the consumption of the inhibitor (40). Therefore, the initial inactivation rates were used to determine the inhibition constant, \( K_i (0.27 \text{ mM}) \), and the rate of inactivation, \( k_{\text{inact}} (0.027/\text{min}) \), for F3Ala from a Kitz-Wilson plot (Ref. 36; Fig. 7c).

Absorption spectra of CGL inactivated by PG or F3Ala showed no decrease of the absorption at 427 nm; i.e. the PLP cofactor in the inactivated enzyme forms a protonated pyridoxaldimine like with its substrate. However, neither with PG nor with F3Ala did dialysis against inhibitor-free buffer lead to a recovery of the enzymatic activity, confirming that an irreversible, covalent modification of the enzyme underlies the inactivation reactions.

**DISCUSSION**

*An Efficient Expression and Purification System for Human CGL*—Detailed mechanistic and structural investigations on human CGL are of prominent importance due to the enzyme’s role in various metabolic disorders and its connection to certain forms of leukemia. We have therefore established the recombinant expression in *E. coli* and a native protocol for the facile production of milligram amounts of homogeneous, active enzyme. This achievement represents the first recombinant purification of a mammalian transsulfuration component.

The native purified protein showed an unexpected UV-visible spectrum with an additional absorption band at 494 nm. The additional 494-nm maximum could be due to some active site heterogeneity maintained throughout the purification with an unidentified *E. coli* factor forming a relatively stable quinonoid PLP derivative. Because the 494-nm absorption band was missing when the holoenzyme was reconstructed from free PLP and apoenzyme, it is unlikely that it indicates different cofactor environments formed within the same protein. The latter phenomenon has been seen with rat liver CGL, where fluorescence studies of PLP-derivatives (41) showed the presence of two differentially designed PLP-binding sites with 10-fold different affinity constants for the cofactor. Similarly, an \( \alpha_2\beta_2 \) composition has been reported for CGL from *E. coli*, in which \( \alpha \) and \( \beta \) signify identical polypeptide chains differing in their net charge and their reaction rates with PG (42).

**Human CGL Displays Strong Substrate and Reaction Specificities**—For yeast CGL, specific activities toward L-cystine and L-cysteine amounting to 78 and 10%, respectively, relative to the L-cystathionine-directed activity have been reported (11). Similarly, the streptomyces enzyme seems to be quite active toward L-cystine (Ref. 1; Table II), and from data published for rat liver CGL similar conclusions can be drawn (14, 17). In contrast, human CGL displays a surprising substrate specificity and a clear preference of C–S over S–S bond breakage; L-cysteine and L-cystine are converted orders of magnitudes more slowly than the natural substrate L-cystathionine. Furthermore, L-cystathionine is split almost exclusively in a CGL-specific manner, in contrast to the behavior of yeast CGL, which also seems to harbor pronounced CBL activity (11). One reason for the failure of human CGL to convert L-cystine could be a high regiospecificity together with the difficulty of polarizing a bond between like atoms, which would hinder the cleavage of the S–S bond under release of L-cysteine. The different behavior of the yeast enzyme indicates a lower regiospecificity that enables the enzyme to attack the C–\( \beta \)-S bond of L-cystine or L-cysteine.

Considering the low in vivo concentrations of L-cysteine and L-cystine (18), it is unlikely that CGL will participate considerably in their in vivo conversion. The present findings therefore call into question the role of CGL in certain forms of cystinosis (accumulation of L-cystine) and the applicability of CGL for cyst(e)ine depletion in order to inhibit the growth of leukemic cells (14).

**Implications for Inhibitor Design Based on AVG**—Because different classes of organisms display different spectra of trans-
sulfuration enzymes, the enzymatic components in plants and microorganisms are attractive targets for the development of antibiotics and herbicides (6, 37), e.g. through interference with methionine biosynthesis. The reactions of transsulfuration enzymes with three mechanism-based inhibitors, AVG, PG, and F3Ala, are summarized in Table IV.

It constitutes a tremendous task to design inhibitors that can distinguish between the homologous active sites of CGL, CGS, and CBL. Any prospective inhibitors should be tested for their reactivity toward CGL, in order to exclude adverse effects in humans. We therefore addressed the reactivity of CGL toward AVG, an antimicrobial agent naturally produced by streptomyces (43). Slow binding inhibition has been documented for AVG acting on *E. coli* and plant CBL, and the molecule was therefore favored as a model substance for the development of novel herbicides (6). In the present analysis, the enzymatic progress curves again show a slow establishment of an enzyme-inhibitor species, and strong inhibition of CGL was observed with AVG concentrations in the order of the total enzyme concentration. AVG therefore qualifies as a slow-tight binding inhibitor also for CGL.

From the proposed scheme for CGL reacting with AVG (Fig. 8b; Ref. 6), a molecular mechanism (mechanism II) can be deduced as follows. In the first step, AVG binds to the active site PLP cofactor by formation of a Schiff base bond. The resulting complex corresponds to a weak $E_1$ complex, because the linkage can be readily resolved by reversion of the transaldimination reaction. Competing with reverse transaldimination, the bound AVG can also face $\alpha$-proton abstraction by the active site lysine and subsequent proton transfer to C-4, leading to a much more stable ketimine complex, corresponding to $E_1^\ast$. This mechanism was proposed by Clausen et al. (6), based on the crystal structure of *E. coli* CBL complexed with AVG. As most of the CBL active site residues are conserved in CGL (Fig. 2), a similar mechanism for the latter enzyme seems likely. A relatively stable ketimine complex also explains the appearance of a 330-nm absorption band under a concomitant decrease of the 427-nm absorption. The ketimine formation is reversible, as was shown by the reactivation of AVG-inhibited CGL accompanied by a decrease of the 330-nm absorption and recovery of the 427-nm absorption. Therefore, the mechanism of inactivation observed for most $\beta,\gamma$-unsaturated inhibitors acting on PLP-dependent enzymes, *i.e.* covalent modification of...
the cofactor or active site residues, can be ruled out for the inactivation of CGL by AVG.

Independent from the mechanistic considerations, the earlier assumption that AVG shows low reactivity against mammalian transsulfuration enzymes (6, 44) is not correct. The overall \( K_i \) (i.e. \( K_{i*} \)) of the AVG-CGL couple (10.5 \( \mu M \)) is only 10-fold larger than that of the AVG-CBL pair (1.1 \( \mu M \); Ref. 6), indicating that AVG shows weak discriminatory power between these two enzymes. Furthermore, AVG still qualifies as a very strong inhibitor of CGL, because \( K_i \) is about 2 orders of magnitude smaller than \( K_m \) for the natural substrate L-cystathionine. AVG therefore seems to be unsuited as a lead compound for the development of antibiotics or herbicides.

**Suicide Inactivators as Lead Substances for Inhibitor Design**—It was thought that mechanism-based inhibition/inactivation (45) might provide the selectivity needed for the development of highly specific inhibitors. Suicide inhibition of CGL and homologous enzymes by F3Ala and PG has been demonstrated previously (7, 39, 46–48). The inhibition reactions showed very low partition ratios, i.e. a strong tendency toward inactivation instead of turnover, rendering these compounds very efficient inhibitors. The substrate and reaction specificity observed for human CGL and the present PG/F3Ala analyses suggest that mechanism-based inhibitors may indeed be developed that are specific enough to distinguish CBL from CGL. PG is a much better inactivator of CGL than F3Ala, and the relative reactivities of the two inactivators qualitatively match those of the L-cystathionine/L-cystine substrate pair (see above). Strikingly, the reactivities of PG and F3Ala are reversed when acting on CBL (Refs. 7 and 22; Table IV). In this

**TABLE IV**

**Inactivation of transsulfuration enzymes**

|        | AVG          | PG          | F3Ala       |
|--------|--------------|-------------|-------------|
| CGL    | Slow binding | Irreversible| Irreversible|
| \( K_i \) | 10.5 \( \mu M \) | ND          | 0.27 \( \mu M \) (\( k_i = 0.027 \text{ min}^{-1}\)) |
| Reference | This work    | This work   | This work   |
| CBL    | Slow binding | No inhibition (E. coli); inhibition (L. lactis) | Irreversible |
| \( K_i \) | 1.1 \( \mu M \) | 0.55 \( \mu M \) (E. coli) | ND (\( k_i = 1 \text{ min}^{-1}\)) |
| Reference | 6            | E. coli (21); L. lactis (22) | 7 |
| CGS    | Slow binding | Irreversible | Irreversible |
| \( K_i \) | 60 \( \mu M \) | 45 \( \mu M \) (\( k_i = 0.16 \text{ min}^{-1}\)) | ND |
| Reference | 8            | 9           | 7           |

\(^a\) ND, not determined.

\(^b\) Value estimated from data given in Ref. 7.
respect it is interesting that γ,γ,γ-trifluoromethylalanine was found to be a much stronger inhibitor of rat CGL than F₃Ala (49), corroborating the idea to base the design of specific CGL and CGS inhibitors on γ-halogenated α-amino acids. A possible explanation for the higher reactivity of PG compared with F₃Ala with CGL can be deduced from the postulated reaction mechanism (Fig. 8c). After initial α-proton abstraction by the active site lysine residue, which is bound to the PLP cofactor in the internal aldime, a ketimine intermediate absorbing at 330 nm is formed by reprotonation at C-4'. The ketimine intermediate subsequently suffers β-proton abstraction by this lysine residue. Another (not yet identified) active site base abstracts a proton from the inhibitor amino group during the preceding transaldimination and subsequently acts as the proton donor in the breakage of the C-γ–S bond. This base functionality may be exploited by PG to form a covalent enzyme-inhibitor complex through its activated γ-carbon atom (Fig. 8c). In contrast, F₃Ala does not provide a reactive γ-carbon atom, and the only base in the enzyme that may be reactive toward the β-carbon could be the active site lysine (Fig. 8d). In CBL, this lysine residue protonates the leaving group at C-β and thereafter is suitably oriented for the Michael addition, whereas the corresponding CGL lysine presumably protonates C-4' (Fig. 8a) and thus is in an unfavorable orientation for the reaction at Cβ.

Taken together, the present studies suggest that the design of specific inhibitors for PLP-dependent enzymes of the γ-family based on irreversible inhibitors seems more promising than that based on reversible ones. The higher specificity of the former compounds is presumably manifested in the steps following the initial α-proton abstraction. Therefore, the development of inhibitors that discriminate between CGS and CGL seems especially difficult, since both enzymes attack at the C-γ of their substrates. However, inhibition of CGL can be achieved with compounds mimicking the first substrate, different activated forms of homoserine. The different leaving groups used by plant and microbial CGL should allow us to discriminate between these enzymes. A similar strategy should be possible for CGL/CGS distinction, at least for specific inhibition of CGS, and yield potent inhibitors when combined with the enzyme-activated irreversible inhibition approach.

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