Structural Basis for the Inhibition Mechanism of Human Cystathionine γ-Lyase, an Enzyme Responsible for the Production of H2S∗

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Impairment of the formation or action of hydrogen sulfide (H2S), an endogenous gasotransmitter, is associated with various diseases, such as hypertension, diabetes mellitus, septic and hemorrhagic shock, and pancreatitis. Cystathionine β-synthase and cystathionine γ-lyase (CSE) are two pyridoxal-5’-phosphate (PLP)-dependent enzymes largely responsible for the production of H2S in mammals. Inhibition of CSE by DL-propargylglycine (PAG) has been shown to alleviate disease symptoms. Here we report crystal structures of human CSE (hCSE), in apo form, and in complex with PLP and PLP-PAG. Structural characterization, combined with biophysical and biochemical studies, provides new insights into the inhibition mechanism of hCSE-mediated production of H2S. Transition from the open form of apo-hCSE to the closed PLP-bound form reveals large conformational changes hitherto not reported. In addition, PAG binds hCSE via a unique binding mode, not observed in PAG-enzyme complexes previously. The interaction of PAG-hCSE was not predicted based on existing information from known PAG complexes. The structure of hCSE-PLP-PAG complex highlights the particular importance of Tyr114 in hCSE and the mechanism of PAG-dependent inhibition of hCSE. These results provide significant insights, which will facilitate the structure-based design of novel inhibitors of hCSE to aid in the development of therapies for diseases involving disorders of sulfur metabolism.

Gaseous messengers or gasotransmitters including nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H2S) have been shown to be important in a range of biological systems. In particular, H2S has recently gained interest as a mediator of cardiovascular and nervous system functions and in inflammation (1–3). Numerous organisms, including bacteria, archaea, and nonmammalian vertebrates as well as mammals, have been shown to produce and utilize H2S gas as signaling molecule at physiological concentrations (20–160 μM) (4). In mammals, two pyridoxal-5’-phosphate (PLP)5-dependent enzymes, cystathionine β-synthase and cystathionine γ-lyase (CSE), are largely responsible for the in vivo production of H2S. Cystathionine β-synthase is expressed predominantly in the central nervous system, and the regulation of cystathionine β-synthase has been well studied (5), whereas CSE is mainly responsible for the production of H2S outside of the nervous system (3), and its regulatory mechanisms are less well understood.

Natural, nonactive CSE mutations, such as T67I and Q240E (6), are associated with cystathioninuria, a disease condition characterized by accumulation of cystathionine in blood, tissue, and urine, sometimes also associated with mental retardation (7). Two of these disease-linked mutations have recently been studied and shown to weaken affinity for PLP (6). Moreover, induction of endotoxemia, acute pancreatitis, hemorrhagic shock, pulmonary hypoxic hypertension, and diabetes mellitus in animals is associated with increased H2S production due to up-regulated CSE production (1, 2). Further, work using several of these animal disease models have revealed that either pre- or post-treatment of animals with inhibitors of CSE, such as DL-propargylglycine (PAG) or β-cyanoalanine, not only inhibit tissue H2S production but also reduce the severity of the disease state. Albeit carried out in animals, these studies serve to highlight the potential importance of CSE-mediated production of H2S in regulating a number of physiological processes in humans.

Steegborn et al. (27) studied the kinetics of hCSE and reported the activity of this enzyme toward γ-cystathionine,
Structural Basis for the Inhibition Mechanism of Human CSE

L-cysteine, and L-cysteine, and several inhibition studies were performed with CSE inhibitors, including PAG. Structure of the Saccharomyces cerevisiae homolog of hCSE revealed insights into the enzymatic specificity among the different family members (9). Recently, Yang et al. (34) showed that H2S is physiologically generated by CSE and that genetic deletion of CSE in mice markedly reduces H2S levels in the serum, heart, aorta, and other tissues. Mutant mice lacking CSE display significant hypertension and reduced endothelium-dependent vasorelaxation and provide direct evidence that H2S is a physiologic vasodilator and regulator of blood pressure.

We report here crystal structures of human cystathionine γ-lyase in the apo form (apo-hCSE), complexed with PLP (hCSE-PLP) and with the inhibitor PAG (hCSE-PLP-PAG) at 2.4, 2.6, and 2.0 Å resolutions, respectively. This is the first report of any crystal structure of a PLP-dependent enzyme in an open conformation that does not have PLP (or a PLP derivative) bound in the active site. In addition, when compared with other PAG complexes, the PAG in hCSE reveals a unique inhibition mechanism, in which PAG does not bind to PLP but adopts a new position in the active site and highlights the singular importance of Tyr114 in hCSE. Further, the crystal structures together with the biochemical and biophysical characterizations provide deeper understanding of the mechanism of hCSE catalysis. We believe that these findings will assist the development of novel inhibitors of hCSE that may be of clinical use in the treatment of a range of disease states resulting from over-production of H2S.

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—The hCSE gene was cloned into a pET-28-based expression vector incorporating a tobacco etch virus-cleavable N-terminal His tag fusion (pNIC-Bsa4). The plasmid was transformed into Escherichia coli BL21 (DE3) and grown in a potassium phosphate-buffered Terrific Broth medium. Expression of hCSE was induced by the addition of 0.5 mM isopropyl-β-D-1-thiogalactopyranoside, and the culture was grown overnight at 18 °C. Cells were harvested and sonicated in lysis buffer (50 mM sodium phosphate, pH 7.5, 10% glycerol, 0.5 mM tris(2-carboxyethyl)phosphine, 300 mM NaCl, 10 mM imidazole, and Complete EDTA-free protease inhibitor (Roche Applied Science). hCSE was purified on a Hi-Trap chelating nickel column (elution buffer: 50 mM sodium phosphate, pH 7.8, 5 mM imidazole, and Complete EDTA-free protease inhibitor mixture (Sigma). hCSE protein was cleaved in the GST beads with thrombin and eluted from the beads with 20 mM sodium phosphate, pH 7.8, 5 mM β-mercaptoethanol, 50 units of thrombin (Sigma). The eluted protein was further passed through a Superdex 200 gel filtration column using an ÄKTA FPLC UPC-900 system (GE Healthcare) and was concentrated to 5 mg/ml in 10 mM sodium phosphate, pH 8.2, and 1 mM dithiothreitol. It has to be noted that this work was initially started independently by two different research groups using two different expression systems.

Crystallization and Data Collection—Initial crystallization conditions were identified from the JCSG+ and Nextral crystal screens (Qiagen) using sitting drop and hanging drop vapor diffusion methods at room temperature. Crystals were grown by mixing equal amounts of protein solution (10 mg/ml hCSE-PLP complex; 5 mg/ml apo-hCSE in the presence of 10 mM L-cysteine) and reservoir solution containing 15% (v/v) polyethylene glycol 3350, 200 mM ammonium citrate, pH 5.6, for hCSE-PLP complex and 20% (v/v) polyethylene glycol 1000, 0.2 M lithium sulfate, 0.1 M phosphate-citrate, pH 4.2, for the apo-hCSE. All tags were removed prior to crystallization. Regardless of their fusion tags, the proteins used for crystallization experiments were equivalent and showed the characteristic absorption for the PLP-lysine internal aldimine bond. However, the bound PLP of the GST-tagged purified hCSE was most likely lost during crystallization.

For the apo-hCSE, rod-shaped crystals with the smallest dimension measuring ~30 μm were obtained after 3 days. X-ray diffraction data were collected at beamline X12C (National Synchrotron Light Source, Brookhaven National Laboratory) using a Q315 CCD detector (Area Detector Systems Corp., Poway, CA). Plate like crystals of hCSE-PLP appeared after 1 day and continued to grow for 1 week. The hCSE-PLP-PAG complex was obtained by soaking crystals of hCSE-PLP in mother liquid supplemented with 10 mM PAG, incubating for 55 min. Diffraction data for hCSE-PLP was collected at the European Synchrotron Radiation Facility beamline ID29 (Grenoble, France) on a Q315 CCD detector (Area Detector Systems Corp., Poway, CA). For the hCSE-PLP-PAG complex, diffraction data were collected at Berliner Elektronenspeicherring-Gesellschaft für Synchrotronstrahlung (BESSY) beamline BL 14.1 (Berlin, Germany) on a MarCCD detector. 20–25% (v/v) glycerol was supplemented with crystallization condition as the cryoprotectant for all crystals used in the experiments. The crystallographic statistics are given in Table 1.

Structure Solution and Refinement—The structure of hCSE-PLP complex was solved using the molecular replacement method with the program MolRep (8) with the coordinates of S. cerevisiae CSE (9) (Protein Data Bank code 1N8P) as the search model. All four molecules of the asymmetric unit were identified. The resulting model with the electron density map was examined in the program COOT (10), and the necessary manual model building was carried out. Several cycles of map fitting and refinement using the program Refmac5 (11) led to convergence. Translation/libration/screw (TLS) refinement using three TLS groups per monomer were used in the refine-
Structural Basis for the Inhibition Mechanism of Human CSE

ment process (12). The TLS groups were selected using the tlsmd server (available on the World Wide Web). Data in the interval 19.9–2.6 Å resolution were used, and at the end of the refinement the R value was 0.180 (R_free = 0.245) for all reflections. Similarly, the hCSE:PLP coordinates were used to determine the structure of apo-hCSE by molecular replacement using MolRep (8). Four molecules of the asymmetric unit were identified. The model building and refinement were carried out using the programs COOT (10) and CNS (13), which led to the convergence of R factor to 0.221 (R_free = 0.236) for reflections with I > 2σ(I) up to 2.4 Å resolution. For the hCSE:PLP-PAG complex, the coordinates for hCSE:PLP were used to obtain initial phases using MolRep (8). Four molecules were present in the asymmetric unit. Data in the interval 19.9–2.0 Å were used, and at the end of the refinement, the R value was 0.158 (R_free = 0.204) for all reflections. For both hCSE:PLP and hCSE:PLP-PAG complexes and apo-hCSE, noncrystallographic symmetry restraints were applied throughout the refinement.

Size Exclusion Analysis of hCSE—Purified hCSE was maintained in 10 mM sodium phosphate (pH 7.8) at a concentration of 6 mg/ml. hCSE was then diluted with either buffer A (10 mM sodium phosphate, pH 7.8) or buffer B (50 mM sodium citrate, pH 4.0, 10 mM cysteine) to 0.5 mg/ml and incubated at 4 °C overnight. These two samples were then loaded on 16/60 Superdex 200 column and run with buffer A or B, respectively.

Analytical Ultracentrifugation Experiment—The oligomeric state of hCSE was investigated by using a Beckman-Coulter XL-I analytical ultracentrifuge fitted with an eight-hole AN-50 Ti rotor and double-sector centerpieces. Samples used for these experiments were 0.4 ml in volume and a concentration of 0.5 mg/ml in buffer A or buffer B, and the same buffer of 0.44 ml in volume was loaded in the reference sector. The sedimentation velocity profiles were collected by monitoring the absorbance at 280 nm as the samples were centrifuged at 40,000 rpm at 20 °C. Multiple scans at different time points were fitted to a continuous size distribution by using the SEDFIT program (14).

Ion Mobility Mass Spectrometry Analysis—Ion mobility mass spectrometry analysis was carried out on a Synapt HDMS mass spectrometer with 8K quadrupole (Waters, Manchester, UK). The spectra were displayed in Masslynx, and IMS ToF data were displayed in DriftScope. Samples of PLP-bound hCSE and apo-hCSE (~2 mM) in buffer A and buffer B were buffer-exchanged into 50 mM ammonium acetate, pH 6.5, and injected using a nanospray source.

Assay of H2S Production—Kinetics studies were performed using a spectrophotometric assay as described by Stipanuk and Beck (15) with some minor modifications. Each test consisted of 5 μg of the purified hCSE enzyme, 2 mM PLP, and l-cysteine (0.75–3.5 mM) in 1.5-ml cryovial tubes. After incubation at 37 °C, 1% (w/v) zinc acetate was added to trap the evolved H2S gas, and the enzymatic reaction was stopped by the addition of 10% (w/v) trichloroacetic acid. 20 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dye and 30 mM FeCl3 were then added, and the absorbance at 670 nm was measured using a microplate reader (Tecan Systems Inc.). Controls were prepared by adding 10% (w/v) trichloroacetic acid before the addition of cysteine to stop all enzymatic reactions. The amount of H2S produced was determined every 3 min over 30 min and calculated against a calibration curve of sodium hydrosulfide. The initial reaction velocity, V0 (units/mg hCSE, where 1 unit = 1 μmol of H2S produced/min) was then plotted against the cysteine substrate concentration, [S], and fitted against the Hill equation,

\[ V_0 = \frac{V_{max}[S]^{h}}{K_{0.5} + [S]^{h}} \]  

(Eq. 1)

The Hill coefficient, h, was determined from the gradient of the logarithmic plot of the Hill equation,

\[ \log \left( \frac{V_0}{V_{max} - V_0} \right) = h \log [S] + \text{constant} \]  

(Eq. 2)

All curve fitting and regression analysis were performed using the graphics software SigmaPlot (Systat).

IC50 Analysis on PAG—Inhibition of hCSE-dependent H2S production was determined by performing the assay with varying concentrations of PAG. Each measurement was assayed in triplicates. Purified hCSE was first preincubated with 2 mM PLP and varying amounts of the inhibitor (PAG 0–4 mM) on ice for 30 min. This was followed by the addition of 5 mM L-cysteine and incubation at 37 °C for 30 min. The IC50 value was then estimated from a plot of percentage inhibition against PAG concentration.

Isothermal Titration Calorimetry—hCSE protein was concentrated to 41 μM in 20 mM sodium phosphate, pH 7.8. PAG was prepared at 600 μM using the same buffer. PAG is loaded into the syringe and titrated against hCSE-PLP in a VP-ITC microcalorimeter (ITC 200; Microcal) performed under identical conditions. The dissociation constants were determined by least squares method, and the binding isotherm was fitted using Origin version 7.0 (Microcal), assuming a one-site binding model. Heat released Q(i), from the ith injection is as follows.

\[ \Delta Q(i) = Q(i) + \frac{dV}{V_0} \left( \frac{Q(i) + Q(i-1)}{2} - Q(i-1) \right) \]  

(Eq. 3)

All measurements were repeated at least twice for verification.

RESULTS

Overall Structures—The structures of apo-hCSE, hCSE:PLP, and hCSE:PLP-PAG complexes were solved and refined to 2.4, 2.6, and 2.0 Å resolution, respectively (Table 1). The resolution of the hCSE:PLP-PAG complex significantly increased, most likely due to the improved quality and size of the crystal. hCSE exists entirely as a tetramer in the PLP-bound state (hCSE:PLP and hCSE:PLP-PAG complexes) and predominantly as tetramers in the apo state in solution as determined by gel filtration, dynamic light scattering, and analytical ultracentrifugation experiments (supplemental Fig. 1). We also report the observation of a tetramer in the asymmetric unit of the hCSE:PLP complex and hCSE:PLP-PAG complex structures. In the case of apo-hCSE, two dimers in the asymmetric unit form tetramers with symmetry-related dimers. Close contact between monomers of the tetramer are maintained by several hydrogen bonds and extensive hydrophobic interactions.
Structural Basis for the Inhibition Mechanism of Human CSE

TABLE 1
Crystallographic data and refinement statistics

| Data set         | apo-hCSE | hCSE-PLP | hCSE-PLP-PAG |
|------------------|----------|----------|--------------|
| Data collection  |          |          |              |
| Cell axial lengths (Å) | 121.31, 121.31, 125.59 | 105.78, 107.57, 153.31 | 105.35, 107.22, 153.31 |
| Space group      | P4_2     | P2_2,2_2 | P2_2,2_2     |
| Resolution range (Å) | 40.0–2.4 (2.5–2.4) | 30.0–2.6 (2.7–2.6) | 20.0–2.0 (2.1–2.0) |
| Wavelength (Å)   | 0.9792   | 1.0332   | 0.9184       |
| Observed reflections | 625250  | 399157   | 739757       |
| Unique reflections | 70985   | 54383    | 109200       |
| Completeness (%) | 99.8 (98.8) | 99.8 (99.3) | 92.9 (98.9) |
| Overall (R/| 14.2 (2.1) | 7.2 (3.4) | 13.8 (3.2) |
| R_free (%)       | 0.127 (0.676) | 0.140 (0.517) | 0.123 (0.445) |
| Solvent content (%) | 52.2    | 47.2     | 49.6         |
| Refinement and quality |        |          |              |
| Resolution range (Å) | 20.0–2.4 | 19.9–2.6 | 19.9–2.0     |
| R_work (%)       | 0.221 (66,936) | 0.180 (51,628) | 0.158 (98,278) |
| R_free (%)       | 0.236 (3582) | 0.245 (2718) | 0.204 (5460) |
| Root mean square deviation bond lengths (Å) | 1.38  | 1.64     | 1.37         |
| Root mean square deviation bond angles (degrees) | 1.38  | 1.64     | 1.37         |
| Average B-factors (Å²) (no. of atoms) |          |          |              |
| Protein atoms    | 50.7 (10,633) | 24.8 (11,846) | 17.4 (11,875) |
| Ligand atoms     |          |          |              |
| PLP              | NA^d     | 31.2 (45) | 16.5 (45)    |
| PAG              |          | 26.5 (24) |              |
| Water molecules  | 42.9 (468) | 22.2 (157) | 27.9 (1159)  |
| Ramachandran plot|          |          |              |
| Most favored regions (%) | 86.2     | 89.6     | 90.1         |
| Additional allowed regions (%) | 12     | 9.5      | 9.0          |
| Generously allowed regions (%) | 0.6    | 0.6      | 0.4          |
| Disallowed regions (%) | 1.3     | 0.3      | 0.4          |

^a R_work = Σ[Io − (|Fo|)]/Σ|Io|, where I is the intensity of the i-th measurement, and (|Fi|) is the mean intensity for that reflection.
^b R_free = Σ[|Fo| − |Fc|]/Σ|Fo|, where Fi and Fo are the calculated and observed structure factor amplitudes, respectively.
^c R_free calculated as for R_work but for 5.0% of the total reflections chosen at random and omitted from refinement for all data sets.
^d Not applicable.

Structures of the individual monomers of apo-hCSE, hCSE-PLP, and hCSE-PLP-PAG are very similar (root mean square deviation for apo-hCSE versus hCSE-PLP is 1.5 Å for 336 Cα atoms; apo-hCSE versus hCSE-PLP-PLP is 1.6 Å for 336 Cα atoms; hCSE-PLP versus hCSE-PLP-PAG is 0.3 Å for 390 Cα atoms) except for two loops near the active site region and a disordered loop at the monomer interface of the dimer in the apo-hCSE. Each hCSE monomer consists of two domains: 1) a larger PLP-binding domain (residues Alaa9–His263) and 2) a smaller domain (residues Val1264–Ser401) (Fig. 1A). The PLP binding domain consists of an α/β/α fold, a seven-stranded mixed β-sheet flanked on three sides by a total of eight α-helices. This central β-sheet consists of seven, mostly parallel, β-strands (β1 ⊥ β7 ⊥ β6 ⊥ β5 ⊥ β4 ⊥ β2 ⊥ β3 ⊥ ) with only strand B7 being antiparallel to the rest. The small domain consists of a four-stranded antiparallel β-sheet (β8 ⊥ β9 ⊥ β11 ⊥ β10 ⊥ ) and three helices on one side of the β-sheet.

Sequence and Structural Similarity—CSE from various organisms forms a conserved group of PLP-dependent enzymes belonging to the family of cystathionine synthase-like proteins. In mammals, highest sequence similarities (80% sequence similarities among the 26 mammalian sequences compared) were obtained from CSE, hypothetical CSE, or CSE homologs. A search for structurally similar proteins was carried out with DALI (16) and BioXGEM (17, 18). Most of the structural similarity was found around the active site region of CSE and the corresponding region in several PLP-dependent enzymes (supplemental Fig. 2).

Active Site—The PLP binding site of hCSE is formed mainly by residues from the central seven-stranded β-sheet as well as α-helices that pack against one face of this sheet (Fig. 1A). PLP is anchored by strong hydrogen bonding between the phosphohosphate moiety and residues contributed by neighboring subunits A (Gly90, Leu91, Ser209, and Thr211) and B (Tyr60 and Arg62) (supplemental Fig. 3). In addition, there are two other hydrogen bonding interactions (by Asp187 and Lys212) with PLP. Lys212 binds PLP, and Tyr114 exhibits aromatic π-stacking interactions to the pyridine ring of PLP (red color in Fig. 1, b and c). Such interactions have also been reported in a number of other PLP enzyme complexes. Fig. 1d shows the omit maps of PLP from the hCSE-PLP structure. Several CSE mutations are associated with cystathioninuria, two of which (T67I and Q240E) have recently been characterized (6). Both mutants have been reported to have greatly reduced enzymatic activities relative to the wild-type enzyme. This reduction in activity has been attributed to lower affinity of PLP with the mutant enzymes. However, in our structure, the importance of these residues in PLP binding is not readily apparent, since they are not directly in contact with PLP.
fold back on the cofactor, thereby establishing effective interactions. This motion shifts the main chain of the central part of both loops at Tyr\textsuperscript{114} and Lys\textsuperscript{212} by as much as 8.6 and 7.0 Å, respectively. The largest displacements are those of the side chains of Tyr\textsuperscript{114} and Lys\textsuperscript{212}, the tips of which move over 19.3 and 10.6 Å, respectively, with a simultaneous large movement of nearby side chains (supplemental movie 1). In the hCSE-PLP complex, there is a two-turn helix (residues Gly\textsuperscript{115}–Tyr\textsuperscript{120}) near Tyr\textsuperscript{114} that unwinds and becomes a loop in the apo-hCSE (Fig. 1b). The distance between the two loops in the open form is 22.7 Å, whereas the same distance in the closed form is 12.7 Å. In the hCSE-PLP complex, Lys\textsuperscript{212} is covalently bonded to the PLP cofactor through the formation of a Schiff base between the carbonyl group of PLP and the amino group of the Lys\textsuperscript{212} side chain (Fig. 1c). However, in the apo structure, Lys\textsuperscript{212} was found to have moved away from the PLP binding site. In the superimposed model of the structures, the distance between PLP of the hCSE-PLP complex and Lys\textsuperscript{212} of the apo structure is too large (11 Å apart) for nucleophilic attack and subsequent formation of the Schiff base. Previously, crystal structures of another PLP-dependent enzyme, namely threonine synthase from A. thalliana, were reported in the PLP-bound and PLP-free states, but no main chain conformational changes were observed in the active site region (19, 20). Similarly, in the hCSE-PLP complex, one of the four monomers of the asymmetric unit is found in the closed conformation without PLP. This indicates that the PLP-free enzyme is highly dynamic and exists as an ensemble of multiple conformational states.

In the hCSE-PLP structure, the N-terminal domain extends to the adjacent monomer and forms part of the neighboring PLP binding site. Residues Tyr\textsuperscript{60} and Arg\textsuperscript{62} electrostatically interact with the phosphate group of PLP (supplemental Fig. 3). However, in the apo-hCSE structure, the electron density map corresponding to this region (Asp\textsuperscript{28}–Ser\textsuperscript{63}) is disordered and indicates the flexibility of this segment due to the absence of PLP. Another missing region in apo-hCSE is from Thr\textsuperscript{355} to Val\textsuperscript{366}; in the hCSE-PLP complex, this region does not directly interact with PLP, but it could stabilize one side of the PLP binding pocket. In the apo-hCSE, the Met\textsuperscript{110}–Asn\textsuperscript{118} loop region occupies the Thr\textsuperscript{355}–Val\textsuperscript{366} region of the hCSE-PLP complex. This is one possible reason why Thr\textsuperscript{355}–Val\textsuperscript{366} of apo-hCSE is shifted further away from the PLP binding site and hence is disordered. l-cysteine needs to bind with PLP to eliminate the SH group to produce H\textsubscript{2}S; therefore, without PLP, hCSE is unable to produce H\textsubscript{2}S.

**Oligomeric States of hCSE**—In order to analyze the oligomeric states of apo- and PLP-bound hCSE, we performed size exclusion chromatography and analytical ultracentrifugation experiments. To best mimic the apo and PLP complex, two buffer conditions were chosen. In buffer A (10 mM sodium phosphate, pH 7.8), which favors the PLP-bound state, hCSE eluted predominantly as a tetramer. In contrast, hCSE exists as a mixture of tetramer and monomeric states in buffer B (50 mM...
structures of both states by x-ray crystallography (Fig. 2, c and d). Taken together, our observations suggest that apo-hCSE exists as a weaker tetramer compared with PLP-hCSE complex in solution.

In Vitro Generation of Apo-hCSE—The bound PLP can be removed from hCSE after 3 days of incubation with 10 mM L-cysteine and 20 mM sodium phosphate, pH 7.8, and subsequent buffer exchange to 20 mM sodium phosphate, pH 7.8, using fast protein liquid chromatography gel filtration (Fig. 3a, red line). However, upon the addition of equimolar PLP, the 427 nm peak reappears within a minute (Fig. 3a, cyan line), indicating that the PLP binding ability of the generated apoenzyme is still retained and the holoenzyme can be regenerated. Further, the characteristic 388 nm peak corresponding to free unbound PLP is absent in this spectrum. This suggests that incubation of L-cysteine with hCSE not only breaks the internal aldime but also facilitates the dissociation of PLP from the enzyme. Notably, L-cysteine was present in the crystallization conditions for apo-hCSE, and no electron density was observed for PLP. It is worth mentioning that apo-hCSE can be generated even at physiologically relevant 2 mM L-cysteine concentrations.

hCSE-PLP-PAG Complex—PAG has been shown to inhibit H$_2$S production by CSE in a number of animal models of disease. For example, PAG accelerated the recovery of blood pressure after a period of hemorrhagic shock in the rat (23). Furthermore, PAG prevented the increase in plasma levels of markers of liver and pancreas injury and reduced the tissue content of myeloperoxidase (an index of tissue neutrophil infiltration) (24). In a model of cecal ligation and puncture, PAG is the most widely used in vivo inhibitor of H$_2$S production by CSE. We performed ITC experiments to measure the binding affinity of PAG to hCSE. The protein was concentrated to 41 $\mu$M, and PAG was prepared at 600 $\mu$M. The number of binding sites ($N$) is 0.993 ± 0.151. Binding affinity ($K_c$) is 3.15 ± 0.35 × 10$^5$ M$^{-1}$, determined using a one set of binding sites model. $\Delta H$ is $-1.24 ± 0.03 × 10^4$ cal/mol, and $\Delta S$ is $-16.2$ (cal/mol)/degrees (supplemental Fig. 4a). Previous experiments to determine the in vitro inhibition potency of PAG had mainly been performed on the inhibition of L-cystathionine to L-cysteine conversion by rat liver (26) and hCSE (27). In this work, we have tested the in vitro inhibition of H$_2$S production from hCSE and determined the IC$_{50}$ of PAG to be 0.2 mM (supplemental Fig. 4b).

To understand the mechanism underlying the effect of PAG on CSE, we have determined the structure of the
hCSE/PLP/PAG complex. In the crystal structure of the hCSE/PLP/PAG complex, the entire structure and the active site region are identical to the hCSE/PLP complex. Lys212 remains covalently attached to C4 of PLP, and Tyr114 mediates stacking interactions with PLP (Fig. 4a). However, C of PAG is covalently bound to Tyr114, and PAG becomes a vinylether. The carboxyl group of PAG forms several hydrogen bonds (3.2 Å) with Arg119 and Arg62 from the adjacent monomer. Further, the amino group of vinylether is hydrogen-bonded to Glu339. The vinylether is positioned such that C is extended to-

FIGURE 3. Generation of apo-CSE and kinetics of H₂S production. a, cysteine incubation with hCSE induces PLP loss. Before cysteine incubation, hCSE (GST-tagged, after cleaving off tag) shows a characteristic internal aldimine peak at 427 nm (black line). After 3 days of cysteine incubation, the protein is buffer-exchanged (gel filtration) to buffer A, and the 427 nm peak is almost unobservable (red line). A cyan line shows the subsequent addition of equal molar PLP. The A₄₃₀/A₂₈₀ ratios are 0.1, 0.02, and 0.09, respectively. The hCSE used for this assay is a GST-tagged construct with the tag removed. b, plots of reaction velocity (V₀) (expressed as units/mg hCSE, where 1 unit = 1 μmol of H₂S produced/min) versus L-cysteine concentration, [S]. The curve was drawn according to the Hill equation. c, logarithmic Hill plot of log(V₀/Vₘₐₓ) against log[S]. Plots of reaction velocity versus substrate concentration suggested the possibility of cooperative binding of L-cysteine to hCSE, hence reflecting the tetrameric state of the enzyme. The maximum reaction velocity, Vₘₐₓ, and substrate concentration at which half Vₘₐₓ is attained, K₀.₅, were 0.1 unit/mg hCSE and 2.8 mM, respectively. The Hill coefficient, h, was 2.6. The turnover number, kₘₐₓ, and the catalytic efficiency (kₘₐₓ/Kₐ₀) for production of H₂S from hCSE using L-cysteine as a substrate were calculated to be 0.4 mol of H₂S produced/mol of hCSE s⁻¹ and 1.5 ⋅ 10⁵ M⁻¹ s⁻¹, respectively.

FIGURE 4. Stereoview of PAG-hCSE active site (a) and superimposed PAG complexes (b). PAG, PLP, and nitrate ion are shown in a thick line. hCSE-PAG, MGL-PAG, and CsdB-PAG (29) are colored green, gray, and pink, respectively. Residues interacting with PAGs and nitrate ion are shown. c, stereoview of the 2Fo – Fc simulated annealing omit map of PAG, Tyr¹¹⁴ from hCSE-PLP-PAG. All atoms within 3.5 Å of PAG and Tyr¹¹⁴ were omitted prior to refinement. The map was contoured at a level of 1.0σ. This figure was prepared using the program PyMol (32).
ward the internal aldimine. In addition, a well defined electron density possibly for a nitrate ion is located inside the active site. It makes several hydrogen bonding contacts (<3.2 Å) with Asp161, Lys212, Ser340, and Arg375. This nitrate ion is most likely acquired during expression and purification stages and retained in the crystal. Fig. 4c shows the electron density map of the bound vinyl ether molecule.

DISCUSSION

A number of human disease states have been shown to involve an imbalance in endogenous H₂S levels. The part played by H₂S in disease has been the subject of several recent reports, which reflects the considerable interest shown in this novel gaseous messenger molecule (1, 2). CSE catalyzes the conversion of L-cystathionine to L-cysteine in the transulfuration pathway and has been suggested to be responsible for endogenous production of H₂S, which fulfills potentially important roles in the cardiovascular and other systems.

An earlier report (22), however, showed only marginal reactivity of hCSE toward L-cysteine. Given the critical role of L-cysteine in changing the conformation of hCSE in our work, we further investigated the ability of hCSE in catalyzing the production of H₂S from L-cysteine. Indeed, this β-lyase reaction is evident for hCSE, albeit about 20 times slower than the cleavage of the C-γ-S bond in the conversion of L-cystathionine to L-cysteine (Fig. 3, b and c, and Table 2). This suggests that L-cysteine is a substrate for hCSE.

Supplemental Fig. 5 shows the likely mechanism for the production of H₂S from L-cysteine. The yellow color of the purified hCSE reflects the presence of PLP bound to hCSE via the active site Lys212 residue. Upon the addition of L-cysteine, the internal aldimine is most likely cleaved off, and possibly L-cysteine binds to PLP, as supported by the loss of yellow color and disappearance of the 427 nm peak (data not shown). Binding of the substrate to PLP in the first step of the reaction is a common feature for all PLP-dependent enzymes (28). Subsequent release of H₂S most probably occurs after a series of electron transfers and cleavage of the C-β-S bond of the bound L-cysteine (supplemental Fig. 5, Step 3).

It has been demonstrated that inhibition of CSE alleviates symptoms of both cardiovascular and inflammatory disease. PAG has been used extensively as an inhibitor of H₂S production and is effective in several animal disease models (1). However, no structural basis has been identified for the mode of binding of PAG to CSE. Our structural characterization of open (apo) and closed (hCSE·PLP and hCSE·PLP·PAG) forms of hCSE, combined with biophysical and biochemical studies, provide novel insights into the mechanism of hCSE inhibition. In particular, the crystal structure of the PAG complex shows a unique internal aldimine that is not observed in other PAG complexes.

To date, there are two other protein-PAG complex structures available, methionine γ-lyase (MGL)-PAG (Protein Data Bank code 1e5e) and CsdB-PAG (29) (Protein Data Bank code 1i12). Fig. 4b shows the superimposition of the active site regions of these PAG complexes. MGL catalyzes a γ-elimination reaction of L-methionine to produce α-ketobutyric acid, methanethiol, and ammonia, whereas CsdB catalyzes both cysteine desulfuration and selenocysteine deselenation. These three proteins belong to the cystathionine synthase-like family (SCOP), and the architecture of the active sites is very similar.

PAGs in CsdB-PAG and MGL-PAG are identical in orientation, and both amino groups of PAG are covalently bound to C4’ of PLP. Interestingly, hCSE-PAG shows a completely different orientation of PAG and reveals an internal aldimine instead (Fig. 4b). In MGL, Cγ of PAG is covalently bound to Tyr111 (Tyr114 equivalent in hCSE); however, in the CsdB structure, the equivalent residue of Tyr114 (His123) is not covalently bonded to Cγ of PAG. Although His123 occupies the same position of Tyr114 and could potentially attack Cγ of PAG, the side chain of His123 is not within close proximity for covalent bond formation. The closest distance between His123 and PAG is 4.14 Å (Ne2 of His123 to CB of PAG; Cγ-C8 is disordered in this structure), whereas for hCSE and MGL complexes, the equivalent distances between Tyr114 hydroxyl group and CB of PAG are more favorable for nucleophilic attack (2.51 and 2.61 Å, respectively). Nonetheless, PAG is covalently attached with C4’ of PLP in CsdB. It is known that PAG is an irreversible inhibitor for hCSE and MGL but not for CsdB (29, 30). We suggest that covalent binding to Tyr114 or Tyr111 (but not with PLP) is a key feature for the irreversible inhibition of hCSE and MGL by PAG.

hCSE-PAG complex shows a unique internal aldimine with PAG. Although MGL and hCSE have similar active site architectures, PAG in hCSE does not bind to PLP and occupies a different position in the active site (Fig. 4b). In MGL, PLP is trapped following PAG binding to PLP and formation of a covalent bond with Tyr111. However, in hCSE, it is most likely that the reaction progresses by the formation of the internal aldimine in tandem release of the PAG from PLP. PAG Cγ remains attached with Tyr114 and is rotated 180°, with its amino group and carboxyl group hydrogen-bonded to Arg62 (from adjacent monomer), Glu339, and Arg119. However in the case of MGL, the corresponding residues are Arg28 (from adjacent monomer), Val136, and Ala116, and no rotation of the PAG was observed.

From the above analysis on PAG enzyme complexes, we identify eight residues that interact with PAG, including Arg62, Tyr114, Arg119, Asp161, Lys212, Ser340, Glu339, and Arg375. Of these, Arg119 and Glu339 are conserved only in CSE homologs, whereas the rest of the PAG-interacting residues are highly conserved in the cystathionine synthase-like family of enzymes (supplemental Fig. 2). In fact, the former is involved in cystathionine distal group binding (9), whereas the latter is involved in stabilizing the carboxyl group of α-carbon of substrate or inhibitors. This difference in primary sequence suggests a basis for the unique function seen in the two classes of enzymes. Further, we believe that the residues Arg119 and Glu339 may be of critical
importance for specific inhibitor design, since they are conserved only in CSE proteins.

Mode of Inhibition—Previous kinetic studies (27) on the inhibition of hCSE by PAG had suggested that the α-amino group of PAG is first deprotonated by Arg62 of the adjacent monomer (B) (Step 1) for transaldimination to occur (Step 2). Lys212 then abstracts a proton from the β-position of the bound alkyne to generate an activated allene (Step 3), which is then attacked by the hydroxyl group of Tyr114 (Step 4) to produce a vinyl ether. Subsequent transaldimination with Lys212 (Step 5) regenerates the internal aldime.

The hCSE-PLP-PAG complex crystal structure provides insights into the inhibition mechanism of hCSE. First, it suggests that the hydroxyl group of Tyr114 serves as the base in step 4 to convert the activated allene to a vinyl ether. Second, we believe that the basic amino acid necessary for deprotonation (step 1) of the incoming PAG and protonation (step 3) of bound alkyne is the proximal Arg62 from an adjacent monomer. Arg62 is located at the entrance of the substrate/inhibitor binding pocket and is in a favorable position to deprotonate the incoming substrate/inhibitor. Additionally, Arg62 (NH2) is 3.3 Å distant from the Cγ of PAG to facilitate the protonation (step 3).

Previously, Steegborn et al. (27) showed no decrease in adsorption at 427 nm upon the addition of PAG and proposed that PLP forms a protonated paradioxalimine similar to PAG. However, in the hCSE-PLP-PAG crystal complex, PAG does not bind to PLP. Further, we verified that there is no change in the spectral property after adding PAG. This is consistent with step 5 of our proposed mechanism, in which transaldimination with Lys212 regenerates the internal aldime.

The bound PAG occupies the space of the side chain of the substrate, thereby inhibiting CSE by blocking accessibility of substrate to the active site via steric hindrance. Additionally, PAG covalently binds and traps residue Tyr114, which is believed to facilitate the release of substrate (31).

Molecular details of PAG-hCSE interactions could not be predicted based on existing knowledge on PAG complexes. Present structural analysis has highlighted the singular importance of Tyr114 in hCSE and its unique specific covalent interaction with PAG, which forms the basis of PAG-dependent inhibition of hCSE. Such a novel strategy forms a new basis to design and develop new drug lead compounds to control the production of H2S.

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