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Structural Snapshots of an Engineered Cystathionine- γ -lyase Reveal the Critical Role of Electrostatic Interactions in the Active Site

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Abstract

Enzyme therapeutics that can degrade L-methionine (L-Met) are of great interest as numerous malignancies are exquisitely sensitive to L-Met depletion. To exhaust the pool of methionine in human serum, we previously engineered an L-Met-degrading enzyme based on the human cystathionine- γ -lyase scaffold (hCGL-NLV) to circumvent immunogenicity and stability issues observed in the preclinical application of bacterially derived methionine- γ -lyases. To gain further insights into the structure–activity relationships governing the chemistry of the hCGL-NLV lead molecule, we undertook a biophysical characterization campaign that captured crystal structures (2.2 Å) of hCGL-NLV with distinct reaction intermediates, including internal aldimine, substrate-bound, gem-diamine, and external aldimine forms. Curiously, an alternate form of hCGL-NLV that crystallized under higher-salt conditions revealed a locally unfolded active site, correlating with inhibition of activity as a function of ionic strength. Subsequent mutational and kinetic experiments pinpointed that a salt bridge between the phosphate of the essential cofactor pyridoxal 5'-phosphate (PLP) and residue R62 plays an important role in catalyzing β - and γ -eliminations. Our study suggests that solvent ions such as NaCl disrupt electrostatic interactions between R62 and PLP, decreasing catalytic efficiency.

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Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.6b01172. Different conformation at the active site for CGL-NLV (Figure S1), structural alignment between the hCGL-NLV crystal found under condition B (green) and apo-hCGL (white, PDB entry 3ELP) showing that these two structures are quite similar (Figure S2), UV–vis scanning of CGL-NLV showing no differences after 14 days at 4 °C (Figure S3), enzymatic activity of CGL-NLV against methionine under different conditions similar to crystallization conditions (Figure S4), salt dependence of the NLV-Y114F variant (Figure S5), and sequence alignment of members of the CGS-like protein family (Figure S6) (PDF)

Accession Codes

Coordinates and structure factors have been deposited in the Protein Data Bank as entries 5TT2 and 5TSU.

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Notes

The authors declare no competing financial interest.

Graphical abstract

L-Methionine (L-Met) depletion has been actively pursued as a cancer treatment for many years, as many malignant human cell lines and tumors are highly sensitive to L-Met depletion compared to normal cells.^{1–5} Tumor cells can display such a strong demand for L-Met that C11-methionine positron emission tomography (MET-PET) has been used to image neoplasms such as glioblastomas, astrocytomas, and metastases to the brain such as melanoma.^{6–8} One aspect of the excessive L-Met demand in tumor cells is the high proliferation rates because L-Met is required for protein synthesis, as a methylation source for DNA and other molecules, and also for polyamine synthesis, a known contributor to tumorigenesis.^{9–11} Because of this strong demand, when L-Met levels are depleted from the normal human serum concentration of $\sim 30 \mu\text{M}$ ¹² to a threshold of $\sim 5 \mu\text{M}$, many tumors cannot survive while normal tissue is much less sensitive.¹³ A significant reduction in serum L-Met concentration has been realized by the systemic application of a methionine- γ -lyase enzyme from *Pseudomonas putida* (pMGL) that degrades L-Met to α -ketobutyrate, methanethiol, and NH_3 , which drastically inhibits tumor growth in a number of animal models.¹⁴ However, while systemic L-Met depletion holds great clinical promise, the advancement of pMGL as a therapeutic has been severely hindered by two major problems. First and most importantly, pMGL is highly immunogenic,¹⁵ and substantially more so than other heterologous enzymes [e.g., the *Escherichia coli* asparaginase II (Oncospar) used to deplete serum L-asparagine to treat acute lymphoblastic leukemia (ALL)]. Second, the enzyme rapidly loses its PLP cofactor and hence activity, both *in vitro* and *in vivo* ($T_{0.5} = 2 \pm 0.1 \text{ h}$), and therefore, impractical dosing schemes (e.g., doses in excess of 100 mg/kg every day) must be used to achieve therapeutically relevant reductions in serum L-Met levels. Thus, despite the outstanding potential of pMGL as an antineoplastic agent, its development has stalled because of its high immunogenicity and poor pharmacodynamics.

To circumvent the significant limitations of bacterial MGLs for human therapy, we previously reported the development of a human methionine- γ -lyase by engineering the

structurally and mechanistically related human enzyme cystathionine- γ -lyase (hCGL) to accept L-Met as a substrate. Both MGL and CGL belong to a superfamily of proteins called CGS-like proteins, which make up a subfamily of type I PLP-dependent enzymes. These enzymes share the same catalytic mechanism to mediate β - and γ -eliminations¹⁶ and similar active site configurations. Although MGLs and CGLs are related in their reaction chemistry, CGL catalyzes the α , γ -elimination of L-cystathionine to L-cysteine, α -ketobutyrate, and NH_3 but has no detectable catalytic activity with L-Met. We previously generated a lead candidate molecule with three active site substitutions, hCGL-E59N-R119L-E339V (called hCGL-NLV), that was able to deplete L-Met *in vitro* and *in vivo* and displayed a half-life 20–30-fold longer than that of pMGL.¹⁷

As part of a campaign to further optimize pharmacodynamic parameters, we undertook a structural and functional study of hCGL-NLV to help direct our engineering efforts. Although the hCGL-NLV variant did not crystallize under the conditions reported for wild-type (WT) hCGL,¹⁸ we found two new conditions that resulted in two different crystal morphologies with two different active site configurations. A comparison revealed a highly similar crystallization environment that differed mainly in ionic strength. Indeed, measuring enzyme activity as a function of ionic strength revealed that both WT hCGL and hCGL-NLV are strongly inhibited by increasing salt concentrations. Suspecting that an important electrostatic interaction was disrupted by solvent ions, we identified active site residue R62, which forms a salt bridge with the phosphate of the PLP cofactor as a likely candidate. Using mutagenesis and chemical modification, we demonstrated that this salt bridge accounts for much of the ionic strength inhibition of enzyme activity. We also obtained structural snapshots of hCGL-NLV during several steps of the reaction pathway, including a Michaelis complex, a reaction intermediate with L-Met in a geminal diamine with PLP, and L-Met in an external aldimine complex. The residues at the outer rim of the active site of hCGL-NLV were identified as determinants for substrate recognition, consistent with its *de novo* ability to accept L-Met as a substrate.

EXPERIMENTAL PROCEDURES

Cloning

An *E. coli* codon-optimized gene encoding human CGL was previously reported¹⁷ and used as a template for constructing the variants used in this study. The hCGL E59N, R119L, and E339V substitutions were introduced by site-directed mutagenesis and subsequently cloned into a pET-28a plasmid with an N-terminal eight-His tag and 3C protease recognition site using ligation-independent cloning.¹⁹ The hCGL-NLV-Y114F, hCGL-NLV-R62A, and hCGL-NLV-R62C variants reported in this study were introduced by site-directed mutagenesis and cloned as described above.

Protein Purification

After the coding regions of the hCGL-NLV variants had been confirmed by sequencing, the resulting plasmids were subsequently transformed into an *E. coli* BL21 (DE3) strain for expression. The expression cultures containing 50 $\mu\text{g}/\text{mL}$ kanamycin were grown at 37 °C until their optical density at 600 nm (OD_{600}) reached 0.6–0.8. The cultures were then cooled

to 16 °C, and protein expression was induced by addition of isopropyl β -D-1-thiogalactopyranoside (IPTG, final concentration of 0.5 mM). Bacterial cultures were harvested after expression for ~12 h by centrifugation at 5000g (Beckman). The resulting cell pellets were resuspended in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 10 mM 2-mercaptoethanol (BME), 10% glycerol, and 0.1% Triton X-100 (buffer A) and lysed by five cycles of sonication (30 s each cycle with 1 s on/off pulses; 2 min pause between cycles) (Misonix sonicator 4000). The resulting whole cell lysate was clarified by centrifugation at 26892g (Sorvall) for 30 min, and the soluble fraction was subsequently loaded onto a nickel affinity column and extensively washed with buffer A containing 50 mM imidazole for 10 column bed volumes. The protein was then eluted with buffer A with an addition of 300 mM imidazole and dialyzed overnight in buffer B [50 mM HEPES (pH 6.5), 150 mM ammonium sulfate, 5% glycerol, 10 mM BME, and 200 μ M PLP]. The recombinant tag was removed by addition of 100 units of HRV 3C protease and incubated at 4 °C overnight. Finally, the protein was further purified by size exclusion chromatography using a Superdex200 column (GE Healthcare) equilibrated in buffer B. Fractions containing hCGL-NLV were pooled, concentrated to ~10 mg/mL, and stored at -80 °C for crystallization and kinetic experiments. All variants reported in this study were purified as described above.

Crystallization and Data Collection

As the human CGL-NLV variant did not crystallize under conditions reported for the WT hCGL protein,¹⁸ novel conditions were screened using a Phoenix Liquid Handling system (Art Robinson) followed by a sparse matrix optimization. Two conditions that resulted in two different crystal morphologies were found from this screen. One form was a sheetlike crystal, found in 30% isopropanol, 150 mM sodium citrate, and 100 mM sodium cacodylate (pH 6.5) (condition A), and crystals with a different rod-shaped morphology were identified in a buffer comprised of 100 mM MES (pH 6.5), 20% PEG3350, 200 mM Na/K tartrate, and 200 mM lithium sulfate (condition B). These crystals were cryoprotected under their crystallization condition with 20% 2-methyl-2,4-pentanediol (MPD) (for the sheetlike crystals) or 30% glycerol (for the rod-shaped crystals) before vitrification in liquid nitrogen prior to data collection. Crystal diffraction data were collected at Advanced Light Source beamline BL5.0.3 (Berkeley, CA) and Advanced Photon Source beamline BL23-ID-B (Chicago, IL). The data were processed using HKL2000,²⁰ and the statistics are summarized in Table 1. A crystal of the NLV-methionine complex was obtained by cocrystallization of the hCGL-NLV protein with L-Met (0.5–1 mM) under condition A at 4 °C.

Structure Determination and Refinement

For the sheetlike crystals of hCGL-NLV obtained under condition A, a significant off-origin peak in the Patterson map of more than 20% of the height of the original peak was observed using the FFT program in the CCP4 suite.^{21–23} This implied the existence of pseudotranslational symmetry (tNCS) in this crystal form. Using the FFT program in the CCP4 software suite, we identified the pseudotranslational vector and further refined it to 0.5, 0.38, 0.5 using the program Phaser in the CCP4 suite.^{21–23} The application of this vector to correct the intensity of the diffraction spots allowed us to identify the correct solution for this crystal form. A model of WT CGL [Protein Data Bank (PDB) entry 2NMP] was used as a search model in the program Phaser from the CCP4 program suite^{21,24} to find

the initial solution. The atomic models were refined using Phenix.refine, reserving 5% of diffraction as an unbiased test set for cross validation (R_{free}).^{25–27} Noncrystallographic symmetry (NCS) restraints were used throughout the refinement.^{27–29} The fitted model was rebuilt using COOT manually, and several iterative cycles of optimization were performed to improve the quality of the model.³⁰ The final models for all the hCGL-NLV structures were evaluated with Procheck³¹ and MolProbity.³² The statistics for refinement are summarized in Table 1. The figures were prepared with PyMol (DeLano, 2002).

Kinetic Assay

Enzyme activity was monitored with a continuous assay using 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as a chromophore to detect the enzyme-catalyzed release of methanethiol and methylselenol from the substrates L-Met and L-selenomethionine, respectively, or for WT hCGL, the release of L-Cys from the substrate L-cystathionine.³³ Steady-state kinetics of hCGL-NLV hydrolysis of L-Met were determined in triplicate using substrate concentrations ranging from 0 to 50 mM. Simple activity was also measured in various buffers: a buffer containing 100 mM MES (pH 6.5) and 50 μM PLP (control), the control with 50 mM ammonium sulfate and 100 mM trisodium citrate (active condition), or the control with 50 mM ammonium sulfate, 133 mM Na/K tartrate, and 133 mM lithium sulfate (inactive condition) at room temperature. The reactions were initiated by adding 50 μL of a substrate DTNB solution (100 mM L-methionine and 165 μM DTNB) to the buffers containing 1 μM protein (final concentration).

For ionic strength-dependent studies, reactions were initiated by adding 50 μL of a substrate DTNB solution (100 mM L-methionine and 165 μM DTNB) together with 45 μL of solutions with varying salt concentrations to 5 μL of pre-aliquoted enzyme at 20 μM (final concentration of 1 μM) in a 96-well Nunc plate (Thermo Fisher). To test the effect of various salt concentrations, enzymatic activity in solutions containing sodium chloride (0–2 M), potassium chloride (0–400 mM), lithium sulfate (0–400 mM), and sodium citrate (0–800 mM) was measured. The raw data and initial rates were obtained and processed using Magellan software (TECAN). Further plotting and nonlinear regression were performed using Origin (Origin-Lab) or KaleidaGraph (Synergy Software, Reading, PA).

Chemical Rescue of hCGL-NLV-R62 Variants with 2-Chloroacetamide

The positively charged thiol labeling reagent 2-chloroacetamide was tested as chemical rescue agent by incubating it with hCGL-NLV-R62C and hCGL-NLV-R62A as a control to determine if a salt bridge could be restored between the phosphate of PLP and an acetamide-labeled R62C residue. A solution was prepared by mixing hCGL-NLV-R62C (or hCGL-NLV-R62A) (final concentration of 20 μM) with 2-chloroacetamide (final concentration of 2 mM) in a buffer comprised of 100 mM HEPES (pH 7.5) and 50 μM PLP and incubated at room temperature for 5 h. After being treated, the protein was then assayed for activity in a solution containing 100 mM HEPES (pH 7.5), 50 mM L-Met, 50 μM PLP, and varying concentrations of NaCl ranging from 0 to 400 mM at a final enzyme concentration of 1 μM using the DTNB assay, as mentioned above.

RESULTS AND DISCUSSION

Active and Inactive Forms of hCGL-NLV Captured in the Crystal Structures

In an effort to gain high-quality hCGL-NLV structures, we screened a variety of matrix conditions as we were unable to obtain crystals of hCGL-NLV by methods reported for WT hCGL.¹⁸ Two conditions for growing diffraction-quality crystals were obtained from this screen. Under one condition, the crystals were found in a sheetlike morphology from a buffer containing 100 mM sodium cacodylate (pH 6.5), 150 mM sodium citrate, and 30% isopropanol (condition A). This crystal form has a space group of $P2_12_12$, with eight monomers (two tetramers) within each asymmetric unit. The second crystals with a rodlike shape were found in a buffer containing 100 mM MES (pH 6.5), 200 mM Na/K tartrate, and 200 mM lithium sulfate with 20% PEG3350 as a precipitant (condition B). These crystals belong to space group $P4_22_1$ with two monomers per asymmetrical unit. Molecular replacement was used to determine the structures of both crystal forms, using WT CGL (PDB entry 2NMP) as the search model. In both cases, the hCGL-NLV structures showed a “dimer of dimers” architecture (Figure 1A) in which two monomers form a functional dimer and two such functional dimers pair up as a tetramer in solution (Figure 1A), as confirmed by gel filtration. The substrate binding pocket is located at the dimer interface with residues from both subunits creating the active site with the essential cofactor pyridoxal phosphate (PLP) covalently linked to K212 located at the tip of an active site loop (residues 210–220) (Figure 1B).

Interestingly, the structures derived from the two different crystal forms showed significant variation at the active site (Figure S1). The structure derived from condition A showed a well-ordered active site, but the structure from condition B exhibited a much higher level of flexibility with residues 355–367 and 28′–63′ from the adjacent molecule being disordered in the model, corresponding to 11.6% of the overall protein. In fact, the structure from condition B displays an open active site architecture virtually identical to that of the reported WT “apo-hCGL” structure (PDB entry 3ELP, rmsd in $C\alpha$ atoms of 0.42 Å) that was crystallized after the removal of PLP in a solution containing 20% (v/v) polyethylene glycol 1000, 0.2 M lithium sulfate, and 0.1 M phosphate-citrate (pH 4.0) (Figure S2). It was reported that apo-hCGL at pH 4.0 exists as a mixture of monomers and a tetramer, suggesting that the absence of the PLP cofactor and low pH weaken interactions between the individual monomers.¹⁸ Thus, we were surprised that our structure resulting from condition B was formed at pH ~6.5 and showed clear PLP electron density extending from the side chain of K212. Furthermore, the covalently linked PLP was stable in hCGL-NLV as the absorbance spectra of the internal aldimine PLP at 412 nm^{34,35} were monitored for 14 days with no obvious changes (Figure S3). We also conducted an *in situ* activity assay of the hCGL-NLV crystal forms by adding substrate L-Met and the color development reagent DTNB directly to the crystallization drops. Only the crystals resulting from condition A produced a dark yellow color that diffused out of the crystals within minutes, indicative of methanethiol production. Thus, we concluded that the inactive hCGL-NLV structures displaying the open active site configuration are not caused by loss of PLP or low pH.

To evaluate the possibility that the lack of activity in condition B was due to crystal packing, we conducted an activity assay by incubating hCGL-NLV in a buffer and salt solution similar to crystallization condition B. We found that the activity of hCGL-NLV toward L-Met is at least 3-fold lower under condition B (pH 6.5 with 200 mM Na/K tartrate and 200 mM lithium sulfate) than under condition A (pH 6.5 with 150 mM sodium citrate) (Figure S4). This reduced activity is consistent with the idea that the buffer constituents from condition B stabilize the inactive open form of the enzyme during crystallization.

Ionic Strength Dependence of hCGL-NLV Form and Function

To identify the factors that promote the active and inactive conformations, we compared the crystallization buffer components. Although the two crystallization conditions have the same pH, they do vary in salt species and concentration (150 mM sodium citrate for the condition giving the active conformation vs 200 mM Na/K tartrate and 200 mM lithium sulfate for the condition giving the inactive conformation). Speculating that ionic strength may play a role in stabilizing inactive conformations and reducing kinetic efficiency, we first measured hCGL-NLV activity as a function of ionic strength using NaCl as a physiologically relevant salt species using the DTNB assay. We found that the relative activity of hCGL-NLV dropped exponentially to near complete inhibition with IC_{50} values of around 34 mM (Table 2 and Figure 2A). Likewise, hCGL-NLV could be completely inhibited by KCl with an IC_{50} of 15 ± 2 mM (Figure 2A). However, titrations of lithium sulfate and sodium citrate showed only 24 and 74% maximal inhibition with relative IC_{50} values of 70 and 76 mM, respectively. To exclude the possibility that the salt-dependent inhibition was introduced through the mutations at E59, R119, and E339, we tested the activity WT hCGL as a function of ionic strength. Similar to the hCGL-NLV variant, WT hCGL is inhibited by increasing salt concentrations with an IC_{50} of 33 mM for NaCl (Figure 2A). In conclusion, the data support the hypothesis that high ionic strength inhibits the enzyme activity of hCGL and its variants.

Because of the marked inhibition as a function of ionic strength, we inspected the hCGL-NLV active site for residues that may be disrupted by solvent ions. In particular, the loop that contains the K212-PLP internal aldimine (residues 210–220) shows dramatic differences between the active and inactive structures (Figure 2B). In the inactive form, the loop is swung outward and the side chain of K212 as well as the PLP cofactor extends away from protein. This places the cofactor out of the active site pocket and far from the other active site residues needed for substrate binding and catalysis (Figure 2C). In contrast, in the active form of the structure of hCGL-NLV, the loop containing K212-PLP is folded back into the substrate binding pocket where the pyridine ring of PLP has an apparent π – π stacking interaction with the phenol side chain of Y114 (Figure 2D). The position of the pyridine ring moiety is further stabilized by hydrogen bonds to the pyridine nitrogen with the carboxyl side chain of D187. The phosphate moiety of the PLP cofactor inserts into the dimer interface and forms a network of noncovalent polar interactions. Significantly, a conserved R62 residue from the adjacent molecule (designated R62') forms a salt bridge with the phosphate group of PLP as well as a hydrogen bond with the phenol of Y114. Additional hydrogen bonds to the PLP phosphate are contributed from Y60', S209, and the backbone amide of L91 that appear to stabilize and orient the cofactor (Figure 2D). The

conformational differences observed in the crystal structures are not crystallographic artifacts as active site loop residues 210–220 are not located close to the crystal packing interface in either crystal form.

The superimposition of the active and inactive structures highlights the fact that the conformational changes cause extensive alteration to the active site. In particular, residue R62' is poised to play an important role in maintaining a polar interaction network by forming a hydrogen bond with Y114 as well as a salt bridge with the phosphate of PLP (Figure 3A). Within the γ -lyase family of enzymes, the conserved Y114 residue participates in the γ -elimination step of the reaction as evidenced by a pMGL Y114F substitution that decreases the $k_{\text{cat}}/K_{\text{M}}$ for L-Met by >1000-fold.^{36,37} To test whether solvent ions disrupt the hydrogen bond between R62' and Y114, we constructed an hCGL-NLV-Y114F variant and assessed the activity as a function of ionic strength. In contrast to pMGL-Y114F, the hCGL-NLV-Y114F variant under low-salt conditions showed an only 5-fold loss of activity compared to hCGL-NLV but was similarly inhibited by NaCl with an IC_{50} of 29 ± 4 mM (Figure S5). Activity tests using alternative substrates such as methylcysteine and cysteine also showed significant inhibition by NaCl, indicating β -elimination reactions are likewise dependent upon ionic strength and inhibition is not due to attenuating the R62' ... Y114 hydrogen bond.

We next constructed and evaluated hCGL-NLV-R62A and hCGL-NLV-R62C variants to remove the salt bridge between R62' and the phosphate of PLP. As expected, both of these variants were highly catalytically impaired with respect to L-Met degradation but did have measurable activity (~1.5% of that of hCGL-NLV) (Figure 3C). To enhance the signal, we used L-selenomethionine (L-SeMet) as an activated substrate because the methylselenol moiety of L-SeMet is a superior leaving group ($\text{p}K_{\text{a}} = 6$) compared to the methanethiol of L-Met ($\text{p}K_{\text{a}} = 10.4$). We compared NaCl inhibition of hCGL-NLV, hCGL-NLV-R62A, and hCGL-NLV-R62C and found the salt inhibition was largely abolished in the hCGL-NLV-R62A and hCGL-NLV-R62C enzymes [IC_{50} values of ~200 mM for both variants compared to an IC_{50} of 23 mM for hCGL-NLV (Table 2)]. As a further confirmation of the importance of an electrostatic interaction between R62' and PLP, we incubated the hCGL-NLV-R62A and hCGL-NLV-R62C variants with 2-chloroacetamidine (CAM), a reagent that forms a positively charged amidino adduct with thiols such as cysteine that resembles arginine (Figure 3B). Incubations with CAM resulted in significant methioninase activity (15% of that of hCGL-NLV) upon addition to hCGL-NLV-R62C but not hCGL-NLV-R62A (Figure 3C), indicating the chemical modification is specific for the R62C substitution. Importantly, CAM-labeled hCGL-NLV-R62C was strongly inhibited by NaCl with an IC_{50} of 25 ± 3 mM, indicating that the enzyme activity is again highly affected by ionic strength (Figure 3D). This strongly indicates that the solvent ion inhibition of hCGL-NLV at the ~30 mM level is caused by locally disrupting the salt bridge between R62 and the phosphate of PLP and taken together with the structural data suggests that very high ionic strength causes additional physical rearrangements.

Salt Dependence of Other CGS-like Family Enzymes

The direct correlation of R62–PLP salt bridge formation and ionic strength inhibition in hCGL and its variants motivated us to inquire if this relationship is found in any other CGS-like family members or other PLP-dependent enzymes. Within CGS-like family members, the active site residues that are important for catalysis are highly conserved: the loop containing the PLP binding lysine residue and an arginine from an adjacent monomer that forms a salt bridge with the phosphate group of PLP and a hydrogen bond with a tyrosine that π stacks with PLP (Figure S6) is found with only few exceptions. For example, the structure of MGL (from *Citrobacter freundii*, PDB entry 3JWA) shows the conserved salt bridge between R60' and PLP (Figure 4A). It has also been reported that the activities of MGL from *Brevibacterium linens* and *Lactococcus lactis* are highly sensitive to salt.^{38,39} MetC and MalY, homologues of CGL and CBL, respectively, from *Lactobacillus casei* containing analogous conserved R62 residues, were reported to be inhibited by high salt concentrations.⁴⁰ Intriguingly, a careful inspection of the MalY structure from *E. coli* (PDB entry 1D2F) crystallized in 100 mM MES/Tris (pH 7.1) and 1.2 M (NH₄)₂SO₄⁴¹ reveals that the loop containing R60' is flipped out from the active site and no longer forms a salt bridge with the phosphate of PLP (Figure 4B). Collectively, within the CGS-like family, it appears that this arginine–PLP salt bridge is responsible for the observed ionic strength inhibition of activity and the local architecture of the active site.

Beyond examples of type I PLP-dependent proteins, D-amino acid aminotransferase (DAAT, PDB entry 4DAA), a type IV fold PLP-dependent enzyme, shows the formation of a salt bridge between PLP and R50 in the active site (Figure 4C) and shows ionic strength inhibition.⁴² In contrast, CysK, which is an *O*-acetylserine sulfhydrylase and belongs to the type II fold PLP-dependent enzyme family, maintains high activity at high salt concentrations.⁴³ This is consistent with the CysK structure from *Haemophilus influenzae* (PDB entry 4HO1) in which no salt bridge with the phosphate of PLP is observed as the Arg of the CGS-like enzymes is replaced with T178 (Figure 4D). Overall, the correlation between the active site Arg–PLP salt bridge and salt-induced inactivation supports the model in which high ionic strength can perturb the salt bridge between PLP and positively charged residues at the active site and lead to the adoption of an alternate inactive conformation. There are other PLP-dependent enzymes that have electrostatic interactions between positive residues and PLP at the active site, but their salt dependence was not evaluated. Our results suggest that their activity may be strongly affected by the environmental ionic strength.

Structure of Substrate-Bound hCGL-NLV

In an effort to gain structural insights into the mechanism of hCGL-NLV-catalyzed L-Met degradation, we obtained the structure of the complex of hCGL-NLV with L-Met (Figure 5). Strong positive electron density was observed at the active site close to the PLP, and subsequent structure building and iterative refinement placed L-Met at the active site in one monomer of the tetramer (molecule A) (Figure 5G). In this structure, the pyridine ring of PLP is tilted toward the phenol ring of Y114. The PLP dihedral angle between the pyridine ring and the Schiff base bond is ~47°, well positioned for attack by L-Met in the Schiff base transfer reaction. The conformation of the active site with bound L-Met also provides the structural basis for the engineered specificity of hCGL-NLV (Figure 5A,B). The residues

mutated in hCGL-NLV (E59N, R119L, and E339V) are located at the outer rim of the substrate binding pocket close to the methanethiol group of L-Met. Residues E59 and E339 are both located at the entrance of the active site and function to coordinate the cysteinyl moiety of L-cystathionine (Figure 5B). Upon an E339V hydrophobic mutation and an uncharged E59N substitution, the L-Met substrate, which is very hydrophobic compared to L-cystathionine, forms more favorable interactions at the active site (Figure 5A). Furthermore, the R119L replacement also provides favorable hydrophobic interactions between the R119L side chain and the L-Met methanethiol group. All three mutations within the hCGL-NLV variant functionally mimic the hydrophobic outer rim of MGL enzymes in facilitating L-Met binding.

Structure of the Geminal Diamine Reaction Intermediate

In another monomer of the tetramer (molecule C), strong positive electron density was observed between the amine of L-Met and the internal aldimine of PLP (~ 1.35 Å), indicating the formation of an additional covalent bond between the amine of L-Met and C4 of PLP (Figure 5C); moreover, the larger dihedral angle ($\sim 54^\circ$) between the pyridine ring and the Schiff base linkage of PLP-lysine also indicates that this is consistent with a geminal diamine intermediate during which PLP is bound to both K212 and L-Met.⁴⁴ Extensive interactions that stabilize this reaction intermediate are formed. The carboxyl group of substrate L-Met forms a salt bridge with R375 (Figure 5D), which is further supported by a hydrogen bond from the side chain of N161 (3 Å) and the main chain amide group of S340. The L-Met side chain is sandwiched among Y60', R62', and Y114, and the triad consisting of the phenol of Y114, the L-Met sulfur atom (3.3 Å), and the guanidinium of R62 (2.8 Å) is well poised to stabilize and protonate the methanethiolate leaving group. The physical location of R62 and Y114 with respect to the methanethiol group and the observation that R62A and R62C substitutions greatly attenuate activity toward L-Met suggest that Y114 donates a proton to the leaving group facilitated by the R62' guanidinium stabilization of the Y114 phenolate ion. When we used L-SeMet as a substrate with the R62A and R62C variants, we observed a 20-fold increase in activity compared to that of L-Met, consistent with a weakened requirement to stabilize and protonate the leaving group (methylselenol pK_a of 6 vs methanethiol pK_a of 10.4).

Structure of the External Aldimine for hCGL-NLV

In another tetramer of the asymmetric unit (molecule E), the electron density between PLP and K212 is missing but a strong positive density was observed between PLP and the substrate, consistent with the formation of an external aldimine with L-Met (Figure 5E). Compared with the internal aldimine structure, the phenol ring of Y114 is tilted further to $\sim 20^\circ$, which now puts the phenol OH group in a hydrogen bond with the γ -sulfur of L-Met (2.5 Å). The formation of this hydrogen bond likely facilitates the subsequent reaction in which Y114 donates a proton to the leaving group and allows the release of the methanethiol product (Figure 5F).

We have captured snapshots of the hCGL-NLV-mediated reaction with L-Met at several key steps: (1) internal aldimine formation between the enzyme and PLP, (2) a Michaelis complex with L-Met, (3) a geminal diamine intermediate, and (4) an external aldimine complex with

L-Met in which the R62' and Y114 residues are primed to facilitate the γ -elimination step to release methanethiol (Figure 6). Our studies also provide a structural rationale for the methionine- γ -lyase activity of hCGL-NLV with comparisons between active and inactive forms of the enzyme identifying an active site salt bridge that promotes γ/β -eliminations, a key interaction that can be disrupted by solvent ions. In light of these experiments, we suggest that a significant rate-limiting step of the hCGL-NLV-mediated reaction is the γ -elimination step in which methanethiol is released from L-Met. This hypothesis is based upon the cocrystal structures in which only L-Met adducts are observed and the finding that activity is significantly enhanced when L-SeMet is used as a substrate. In contrast to the hCGL-NLV active site architecture, the MGL enzymes have an extra loop and additional residues that form a hydrogen bond network in the vicinity of R62' aiding in the γ -elimination of methanethiol. Additional engineering of hCGL-NLV for increased activity may need to focus on adding residues that can further stabilize the leaving group and overcome this apparent kinetic trap.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CGL	cystathionine- γ -lyase
MGL	methionine- γ -lyase
CBL	cystathionine- β -lyase
PLP	pyridoxal 5'-phosphate
MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
PEG	polyethylene glycol
MPD	2-methyl-2,4-pentanediol
CAM	2-chloroacetamide

BME	2-mercaptoethanol
rmsd	root-mean-square deviation

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Figure 1.

Overall architecture of hCGL-NLV. (A) Tetramer formation of CGL. Two monomers (colored green and pink) form a dimer with active sites at the dimer interface (highlighted by yellow circles). Two such dimers form the tetramer. The structure shown is obtained from crystal form 1 (active form found under condition A). (B) Close-up view of the hCGL-NLV variant active site, in which the residues interacting with PLP are shown as sticks. For the sake of clarity throughout, we denote the molecule containing the active site K212 residue with normal numbering and the residues from an adjacent dimer with a prime.

Figure 2.

Ionic strength inhibition and structural differences between the two crystallization forms. (A) Relative activity of hCGL-NLV or WT hCGL as a function of salt concentration: WT hCGL with NaCl (○) and NLV with NaCl (■) and KCl (●). (B) Alignment of monomers from crystal form 1 (white) and crystal form 2 (green) showing significant changes in the orientation of the K212-PLP active site loop. The K212-PLP is shown as sticks. Hydrogen bond network between PLP and hCGL in the (C) inactive configuration and (D) active configuration. The hydrogen bonds are shown as dashed lines. Surface charge potentials are colored with blue as positive charges and red as negative charges with a gradient of 100 kT/e.

Figure 3.

Structural and functional relationship of the R62'-PLP salt bridge of hCGL-NLV. (A) Active conformation of hCGL-NLV depicting the hydrogen bonding network between PLP and residues around it as well as the salt bridge between R62' and the phosphate of PLP and hydrogen bonding between Y114 and R62' (dashed lines). (B) Scheme of chemical modification at R62C that restores the positive charge of residue 62. (C) Mutation of R62 to Ala or Cys results in a significant loss of activity that can be partially restored only to hCGL-NLV-R62C by incubation with 2-chloroacetamide. (D) Restoration of inhibition by NaCl after chemical modification of R62C by 2-chloroacetamide as a function of salt concentration.

Figure 4.

Conserved salt bridge between PLP and arginine in the CGS-like family. (A) Active site of MGL from *C. freundii* (PDB entry 3JWA) with a salt bridge between PLP and R60'. (B) Active site of MalY from *E. coli* crystallized in 1.2 M $(\text{NH}_4)_2\text{SO}_4$ (PDB entry 1D2F). (C) Active site of DAAT from *Bacillus* sp. (PDB entry 4DAA), a type IV fold PLP-dependent enzyme, depicting a salt bridge between Arg50 and PLP from the same monomer. (D) Active site of CysK from *Haemophilus influenzae* (PDB entry 4HO1).

Figure 5.

Substrate- and intermediate-bound forms of hCGL-NLV. Comparison of hydrophobicity around the substrate binding pocket between hCGL and hCGL-NLV. The surface charge potential is colored blue for positive charges and red for negative charges using a gradient of 100 kT/e for (A) hCGL-NLV and (B) WT hCGL. Methionine was docked into the active site of WT hCGL by superimposing hCGL and hCGL-NLV structures, and the mutated residues are shown as sticks. (C) Composite omit map ($2mF_o - DF_c$) of the active site of hCGL-NLV modeled as a geminal diamine intermediate (chain C) and shown as a net at a contour level of 0.9σ . PLP and L-Met were built into the density and are shown as sticks. Two monomers creating the active site are colored green and pink. Two different orientations with a rotation

of 90° are shown with residues shown as sticks. (D) Contacts between the geminal diamine intermediate and residues of hCGL-NLV. The two monomers that form the active site are colored pink and green. The residues participating in substrate and intermediate binding are shown as sticks with polar interactions shown as dashed lines. (E) Composite omit map ($2mF_o - DF_c$) of the active site of hCGL-NLV in the substrate-PLP external aldimine form (chain E) shown as a net at a contour level of 1.0σ . (F) Contacts between the external aldimine and residues of hCGL-NLV. The residues participating in substrate and intermediate binding are shown as sticks with the hydrogen bond between Y114 and L-Met shown as a dashed line. (G) Active site of hCGL-NLV with substrate L-Met binding (chain A). The electron density of substrate methionine is shown as a composite omit map ($2mF_o - DF_c$) at a contour level of 1.0σ .

Figure 6. Reaction pathway of the hCGL-NLV variant mediating γ -elimination reaction. The top panel depicts the reaction scheme with L-Met and the bottom panel the corresponding structures of the hCGL-NLV variant. The two monomers forming the active site are colored green and pink.

Table 1**Structural Determination and Refinement Statistics of hCGL-NLV**

	inactive hCGL-NLV	active hCGL-NLV
Data Collection ^a		
space group	<i>P</i> 4 ₂ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2
cell dimensions <i>a</i> , <i>b</i> , <i>c</i> (Å)	120.2, 120.2, 125.6	163.7, 181.1, 111.9
angles α , β , γ (deg)	90.0, 90.0, 90.0	90.0, 90.0, 90.0
resolution (Å)	50.00–2.95 (3.00–2.95)	50.00–2.20 (2.24–2.20)
<i>R</i> _{sym}	0.151 (0.927)	0.097 (0.817)
<i>I</i> / σ <i>I</i>	7.43 (1.16)	18.78 (2.18)
completeness (%)	97.70 (98.20)	95.9 (96.7)
redundancy	4.4 (4.4)	5.1 (5.0)
Refinement		
no. of reflections	19544	161429
<i>R</i> _{work} , <i>R</i> _{free} ^b	0.2021, 0.2549	0.1711, 0.2016
no. of atoms	5344	24979
macromolecules	5324	23895
ligand/ion	20	72
water	0	1042
<i>B</i> factor (Å ²)	48.50	52.12
protein	48.40	52.44
ligand/ion	80.30	49.43
water	–	44.74
Ramachandran favored (%)	99.41	97.23
Ramachandran outliers (%)	0	0
rmsd for bond lengths (Å)	0.003	0.004
rmsd for bond angles (deg)	0.72	0.70

^aStatistics for the highest-resolution shell are given in parentheses.

^b*R*_{free} is calculated with 5% of the data randomly omitted from refinement.

Table 2Influence of Solvent Ions on the Activity of hCGL-NLV and Other Variants^a

variant	substrate	salt	% maximal inhibition observed	relative IC ₅₀ (ionic strength) (mM)
WT CGL	L-Met	NaCl	100	33 ± 3
NLV	L-Met	NaCl	96	34 ± 3
NLV	L-Met	KCl	99	15 ± 2
NLV	L-Met	Li ₂ SO ₄	24	70 ± 18
NLV	L-Met	Na citrate	74	76 ± 12
NLV	L-SeMet	NaCl	100	23 ± 3
R62A	L-SeMet	NaCl	70	190 ± 22
R62C	L-SeMet	NaCl	95	210 ± 30
R62C with CAM	L-Met	NaCl	100	25 ± 3
Y114F	L-Met	NaCl	95	29 ± 4

^aL-Met and L-SeMet were used as substrates at different salt concentrations. The relative activity was measured, and data were regressed by a single-exponential curve to calculate IC₅₀.