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Biochemical Stability and Molecular Dynamic Characterization of *Aspergillus fumigatus*

Cystathionine γ -Lyase in Response to Various Reaction Effectors

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Highlights

- The conformational stability of *A. fumigatus* CGL for buffers and destabilizers was assessed.
- Cystathionine γ -lyase thermal stability was increased in phosphate and MES buffers, unlike Tris-HCl and HEPES.
- The catalytic stability of CGL was increased by trehalose and glycerol, in contrary to GdmCl.
- Dynamic simulations confirm the overall CGL scaffold stability with flexibility at non active site domains.
- CGL has eight buried Trp residues that can be easily solvent exposed by destabilizer's perturbation.

27 **Abstract**

28 Cystathionine γ -lyase (CGL) is a key enzyme in the methionine-cysteine cycle in all living
29 organisms forming cysteine, α -ketobutyrate and ammonia *via* homocysteine and cystathionine
30 intermediates. Although, human and plant CGLs have been extensively studied at the molecular and
31 mechanistic levels, there has been little work on the molecular and catalytic properties of fungal CGL.
32 Herein, we studied in detail for the first time the molecular and catalytic stability of *Aspergillus*
33 *fumigatus* CGL, since conformational instability, inactivation and structural antigenicity are the main
34 limitations of the PLP-dependent enzymes on various therapeutic uses. We examined these properties in
35 response to buffer compositions, stabilizing and destabilizing agents using Differential Scanning
36 Fluorometry (DSF), steady state and gel-based fluorescence of the intrinsic hydrophobic core, stability
37 of internal aldimine linkage and catalytic properties. The activity of the recombinant *A. fumigatus* CGL
38 was 13.8 U/mg. The melting temperature (T_m) of CGL in potassium phosphate buffer (pH 7.0-8.0) was
39 73.3°C, with $\sim 3^\circ\text{C}$ upshifting in MES and sodium phosphate buffers (pH 7.0). The conformational
40 thermal stability was increased in potassium phosphate, sodium phosphate and MES buffers, in contrast
41 to Tris-HCl, HEPES (pH 7.0) and CAPS (pH 9.0-10.0). The thermal stability and activity of CGL was
42 slightly increased in the presence of trehalose and glycerol that might be due to hydration of the enzyme
43 backbone, unlike the denaturing effect of GdmCl and urea. Modification of surface CGL glutamic and
44 aspartic acids had no significant effect on the enzyme conformational and catalytic stability. Molecular
45 modeling and dynamics simulations unveil the high conformational stability of the overall scaffold of
46 CGL with high flexibility at the non-structural regions. CGL structure has eight buried Trp residues,
47 which are reoriented to the enzyme surface and get exposed to the solvent under perturbation of
48 destabilizers. Furthermore, electrostatic calculations of selected snapshots of CGL 3D structure under
49 different experimental conditions showed a remarkable differences on the polarity of the enzyme
50 surface.

51

52 **Keywords:** *Aspergillus fumigatus*; Cystathionine γ -lyase, Conformational Structure; Electrostatics; Molecular
 53 Dynamics; MMPBSA; Thermal Shift Assay.

54

55 1. Introduction

56 Cystathionine γ -lyase (Cystathionase, CGL, EC 4.4.1.1) is a pyridoxal 5'-phosphate-dependent
 57 enzyme that catalyzes the elimination reactions of cystathionine to cysteine, α -ketobutyrate, and
 58 ammonia [1] in most prokaryotes and eukaryotes [2-4]. CGL and cystathionine β -synthase (CBS) are
 59 the key enzymes in transsulfuration and reverse transsulfuration metabolic pathways for conversion of
 60 cysteine to methionine and *vice versa* through homocysteine and cystathionine intermediates [5]. In
 61 addition to the specificity of CGL towards L-cystathionine it has affinity to eliminate homoserine,
 62 cystine and cysteine to keto acids, NH_3 and H_2S [6-8]. The reverse transsulfuration pathway of
 63 homocysteine to cysteine by CBS and CGL *via* cystathionine intermediate is restricted to fungi and
 64 human [5].

65 CGL deficiency in humans is directly associated with metabolic disorders related to
 66 accumulation of cystathionine (homocystathioninuria, homocystinuria) [9, 10]. Genetic deletion or
 67 decrease in the expression of CGL in mice entails a decrease in the level of cysteine, glutathione (GSH),
 68 taurine and H_2S and subsequently these animals suffer from hypertension [10], cystathioninuria and
 69 hyperhomo- cysteinemia [11]. Also, dysregulation of CGL/ H_2S pathway is implicated in the
 70 pathogenesis of cardiovascular diseases such as atherosclerosis [12], human fetal liver [13] and
 71 vasorelaxation, oxidative stress, vasodilation *via* opening of K_{ATP} channels, causing membrane
 72 hyperpolarization as reviewed by Hua et al. [14]. On the other hand, overexpression of CGL in cells
 73 either *in vivo via* genetic manipulation or *in vitro* supplementation increases the level of GSH and
 74 production of H_2S [15], confirming the roles of CGL in H_2S homeostasis.

H₂S has recently emerged as a gaseous transmitter and inflammation modulator [16] with crucial functions as pivotal signaling molecule, anti-inflammatory, anti-apoptotic and cytoprotective molecule [17, 18] in plants [19], fungi [20] and humans [21, 22]. In human using L-cysteine as substrate, CGL is responsible for the endogenous supply of H₂S outside of the nervous system [23], predominantly in the cardiovascular system, liver, kidney and vascular smooth muscles [24] [16], while CBS is expressed predominantly in central nervous system [7].

Expression of CGL gene (*mecB*) and the level of gene transcripts under nutritional conditions of sulfur starvation in addition to supplementation of sulfur amino acids such as L-methionine and cysteine were studied extensively in fungi [20, 25, 26]. However, a very few studies on the molecular and catalytic properties of CGL from fungi compared to the extensive studies about the human homolog were reported. Also, despite the central metabolic role of CGL in a broad pathological spectrum and its potential applications in various therapeutic aspects, the detailed mechanistic properties of the enzyme from filamentous fungi remain unclear, while the conformational structures of human and yeast CGL have been clarified by crystallography [6, 27]. Although the biochemical properties of non-recombinant CGL from *Neurospora* sp [28], *Aspergillus carneus* [29] was assessed, these studies were hampered by low enzyme purity. We hypothesize that purified recombinant fungal CGL offers a reliable model for biochemical and molecular studies for exploitation on various pharmaceutical and biotechnological applications.

We describe in the following study the molecular cloning and heterologous expression of CGL from *A. fumigatus* in *Escherichia coli* for maximum exploitation in therapeutic application. The availability of the protein in a purified form has allowed us to obtain a deeper insight into its biochemical properties through characterization of its kinetic and spectral properties. The conformational stability of the purified recombinant *A. fumigatus* CGL was assessed by DSF and steady state fluorescence assays. The enzyme folding/unfolding structural stability was studied based on its

intrinsic fluorescence emission. The catalytic stability normalized to the PLP-internal Schiff base in response to various effectors was evaluated from the UV-visible spectra, in addition to chemical targeting of CGL surface amino acids. Molecular modeling approaches (homology modeling, molecular dynamic simulations and free energy calculations) were undertaken to characterize the enzyme *in silico* for interpreting the experimental data and its correlation with the 3D structure.

2. Material and Methods

Materials

L-Cystathionine, SYPRO Orange (5000X in DMSO) and isopropyl β -D-thiogalactopyranoside (IPTG) was purchased from Sigma-Aldrich. *Hind*III and *Bam*HI were purchased from New England Bio Lab. CriterionTM Precast Gels (4-20% Tris-HCl) were obtained from Bio-Rad, USA. The expression vectors, *E. coli* DH α component and *E. coli* BL21 CodonPlus[®] cells were purchased from Invitrogen (Life Technology). All chemicals were of analytical grade.

Methods

2.1 Cloning of CGL from *A. fumigatus*

Aspergillus fumigatus Af293 was used as the source of CGL encoding gene [30]. CGL gene was synthesized as gBlock fragments at Integrated DNA Technologies (Coralville, Iowa, USA) with *Bam*HI and *Hind*III at N-terminus and C-terminus, respectively. The synthesized CGL gene (*mecB*) (XM_742148.1) was used as a template for PCR amplification using the sense and antisense primers (5'-CGCGGATCCGATGACTGCATCTTC-3' and 5'-GGGAAAGCTTTTAAGAAGATCCGT-3'); the underlined bases are the restriction sites for *Bam*HI and *Hind*III, respectively. PCR product was digested with *Bam*HI and *Hind*III and the released fragment was cloned in in frame with T7 and His-tags in the pET-28b bacterial expression vector. The construct was verified by sequencing and transformed into BL21-CodonPlus[®] Chemical Competent Cells.

123

124 **2.2 Expression of CGL and purification of the recombinant protein**

125 Bacterial cultures were grown at 37°C in a shaker at 250 rpm to an OD₆₀₀ of 0.6. The CGL
 126 expression was induced by adding IPTG to a final concentration of 0.5 mM, then the culture was
 127 allowed to grow for another 4 h at the same conditions. Cells were harvested, washed with 50 mM Tris-
 128 HCl (pH 7.5) and re-suspended in buffer A (50 mM Tris, pH 7.5; 150 mM NaCl, 5 mM imidazole, 10
 129 µM pyridoxal 5'-phosphate (PLP), 1mM DTT and 1mM β-mercaptoethanol) supplemented with
 130 protease inhibitor cocktail (Roche Diagnostic, Indianapolis, IN) and lysozyme 200 µg/ml (Sigma-
 131 Aldrich). The suspension was then placed on ice and sonicated three times, 10 s each, using a Virsonic
 132 digital 475 ultrasonic cell disrupter (Virtis, NY). The extract was centrifuged at 16,000 xg for 15 min to
 133 obtain soluble (supernatant) fraction that was used for enzyme purification. Briefly, supernatant was
 134 allowed to bind to His-Bind resin (Novagen, Madison, WI) in the presence of buffer A and washed with
 135 5 volumes of the same buffer but containing 50 mM imidazole. The bound proteins were eluted in
 136 buffer A supplemented with 400 mM imidazole. The eluted fractions (0.5 ml) were checked for
 137 molecular homogeneity by SDS-PAGE and Western blotting using T7-Tag polyclonal antibody alkaline
 138 phosphatase-conjugate following the manufacturer's instructions (Novagen, Madison, WI). The most
 139 active and homogenous fractions were pooled and dialyzed overnight against 20 mM potassium
 140 phosphate buffer pH 7.5 containing 0.5 M NaCl, 10 µM PLP, 1 mM DTT and 1mM β-mercaptoethanol.

141

142 **2.3 Protein concentration and Enzyme activity assay**

143 The protein content was quantified by the Bradford assay (Bio-Rad) using BSA as a standard.
 144 The activity of CGL was determined based on the released α-ketobutyrate [6] with minor modifications.
 145 The reaction mixture contained 500 µl L-cystathionine (1 mM) in potassium phosphate buffer (100 mM,
 146 pH 8.0), 20 µM PLP and 100 µl of the enzyme preparation in 1 ml total volume. After incubation for 10

min at 37°C, 500 µl of 4.5% TCA was added to stop the reaction and then centrifuged for 10 min at 5000 xg. 500 µl of the supernatant was mixed with 500 µl of 3-methyl-2-benzothiazolinone hydrazone (MBHT) reagent (0.05% MBTH in 1 M sodium acetate buffer, pH 5.2), incubated for 30 min at 50°C and the developed color was measured at 320 nm. Standard curve of α -ketobutyrate was prepared under the same conditions. On unit of CGL was expressed as the amount of enzyme releasing 1 µmole of α -ketobutyrate per min and the specific activity was expressed by the enzyme activity in unit per mg protein. The K_m and V_{max} values of CGL were determined from the Lineweaver-Burk plots using Graphpad Prism (Graphpad Software Inc., La Jolla, CA, USA) by plotting the reciprocal of initial reaction velocity V_o (U/mg/min) against L-cystathionine concentrations (0-100 mM).

2.4 Differential scanning fluorometry (DSF) / thermal shift assay

The thermal unfolding of CGL was monitored by DSF assay using the real-time PCR machine (MX3005, Agilent Technologies, Santa Clara, CA) using SYPRO Orange [31, 32]. The enzyme was dialyzed in 10 kDa Slide-A-Lyzer (Thermo Scientific) against 100 mM potassium phosphate buffer (pH 8.0) to remove the residue of DTT and β -mercaptoethanol. The enzyme (45 µl) was mixed with SYPRO Orange (5 µl of 100 X diluted from 5000 X stock solution dissolved in DMSO) and vortexed. The qPCR machine was programmed for 15 min prewarming at 30°C with subsequent temperature increasing from 31°C to 95°C at 1°C/ min ramping. SYPRO Orange fluorescence was monitored using FAM (Excitation 492 nm) and ROX (Emission 610 nm) filters. Data were exported to MxPro software (Mx3005P, Standalone) and the melting temperature (T_m) was calculated as arbitrary fluorescence units (AFU)/ function of temperature increments (dT) [32].

2.5 UV-visible spectral analysis

170 The strength of internal PLP-Schiff base of CGL was determined at 420 nm [5] using Spectra
171 Max M5 (Molecular Devices, Neobits Inc., CA, USA). The free PLP was determined from absorbance
172 at 390 nm using potassium phosphate buffer (100 mM, pH 8.0) as blank baseline [33].

173

174 *2.6 Steady state fluorescence spectral analysis*

175 The purified enzyme was concentrated by 30 kDa Amicon membrane at 10000 xg at 4°C till
176 reduction of ~80% of its initial volume, then pipetted with PLP-free potassium phosphate buffer (100
177 mM, pH 8.0) to 1 ml. The centrifugation cycles were repeated to completely remove the free PLP till the
178 absorbance at 390 nm was ≤ 0.05 . The intrinsic emitted fluorescence of CGL was scanned at 335-340
179 nm and 498-500 nm with a 295 nm excitation [34] using Spectra Max M5. The fluorescence emission at
180 335-340 nm is directly related to the tryptophan residues, while the emission at 498-500 nm is referred
181 to the internal PLP Aldimine linkage of CGL. Spectra of the buffer were measured as baseline at the
182 same excitation and emission wavelengths and then subtracted from the samples spectra.

183 The tautomeric equilibrium of CGL internal PLP from active ketoenamine to inactive enolimine
184 forms was measured at 410 nm and 330 nm excitation, respectively [35]. The emitted fluorescence was
185 scanned from 480-510 nm and from 450-460 nm for ketoenamine and enolimine PLP, respectively [36],
186 using PLP free potassium phosphate buffer. The spectra of buffer at the same excitation and emission
187 wavelengths were subtracted from the sample. All fluorescence measurements were conducted in
188 Microtest™ 96 well plates (BD, Falcon™, Black /Clear Bottom). The unbound PLP of the CGL was
189 assessed from its fluorescence emission spectra at 446 nm by excitation at 353 nm based on authentic
190 PLP [37].

191

192 *2.7 Aggregation and cross linking of CGL*

The molecular mass and cross linking of CGL was monitored by gel-filtration kit (GE Healthcare, Life Sciences), using Sephadex G₂₀₀ column at a flow rate of 0.5 ml/ min and potassium phosphate (50 mM, pH 8.0) as eluent. Ovalbumin (44 kDa), canalbumin (75 kDa), aldolase (158 kDa), ferritin (440 kDa) and thyroglobulin (669 kDa) were used as authentic proteins. Blue dextran was used to monitor the void volume. The concentration of proteins was monitored at A₂₈₀ nm. The size of the enzyme was assessed from its elution volume normalized to authentic protein standards. Molecular aggregation of CGL was assessed by Dynamic Light Scattering (DLS) assay (Nano-ZS, Malvern Instrument Limited, Malvern, UK) [38, 39]. The measured enzyme size by DLS was normalized to the size of standard proteins as above.

2.8 Conformational stability (unfolding/ folding) of CGL

The conformational stability of CGL *via* unfolding of its subunits and exposing the hydrophobic core to surface polar environment was evaluated from its intrinsic emitted fluorescence [40]. *A. fumigatus* CGL has two residues of tryptophan (Trp¹⁵⁷ and Trp²⁵⁴) per subunit that are responsible for its intrinsic fluorescence. The enzyme (100 µl) was incubated with denaturant urea and guanidinium chloride (GdmCl) (0.5-5.0 M) in potassium phosphate buffer (100 mM, pH 8.0) at 37°C for 2 h. The emitted fluorescence for the tryptophan residues was scanned from 335 to 340 nm at 295 nm excitation [34] using Spectra Max Fluorometer. Standard tryptophan was measured under the same excitation and emission conditions. The residual enzyme activity and UV-spectra were assessed. The unfolding ratio ($(F_i - F_o) / (F_d - F_o)$) was determined based on the intensity of emitted fluorescence under denaturing conditions compared to control [34].

2.9 Chemical modification of surface amino acids residues

Glutamic and aspartic acids of CGL was selectively modified at their carboxyl groups by *N*-ethyl-3-*N'*, *N'*-dimethylaminopropylcarbodiimide (EDC) [41]. Lysine amino groups were selectively modified by protonation *via* reaction with *N*-hydroxysuccinimide (NHS) [42]. Serine and threonine were modified by reaction with CNBr [43]. CGL (1.4 U/mg) was gently mixed with EDC, NHS, CNBr (0, 10, 20, 40 mM) in 100 mM potassium phosphate buffer (pH 8.0) at 25°C for 60 min in 1 ml total volume. The residual enzymatic activity was determined as above. The shift on electrophoretic mobility of the modified CGL was assessed by denaturing PAGE, the enzyme cross linking was assessed by gel-filtration and DLS analysis as described above.

2.10 Effect of buffer type/ pH on conformational and catalytic stability of CGL

The conformational and catalytic stability of CGL maintained in various buffers (HEPES, sodium bicarbonate, Tris-HCl, potassium sodium phosphate, potassium phosphate, MES and phosphate buffered saline) were assessed. The buffers strength was 100 mM and the pH was adjusted to pH 6.0, 7.0 and 8.0, while CAPS buffer was used for pH 9.5 and 10.5, using water-dissolved enzyme as a negative control. Briefly, the reaction mixture contains CGL (2.8 U/mg) on the tested buffer was incubated at 25°C for 60 min in a total volume 200 µl. The residual enzymatic activity, thermal conformational stability by DSF, emission of intrinsic fluorescence at 340 nm, internal Schiff base and PLP tautomeric equilibrium were assessed as mentioned above.

2.11 Effect of different denaturants/ Cosolvents on the catalytic and thermal stability of CGL

The effect of urea, GdmCl (0.5, 1.0 and 2.0 M), EDTA (1.0, 5.0, 10.0 mM), DTT (0.1, 0.5, 1.0 mM), Tween 20 (1.0, 5.0, 10.0 mM), pyridoxal 5'-phosphate (0.1, 0.2, 0.4 mM) and Triton X100 (0.1, 1.0 mM) on the conformational stability of CGL was investigated. The effect of cosolvents (trehalose, glycerol and sucrose) and aliphatic alcohols (ethanol, methanol, ethylene glycol, isopropanol, 1,2-propndiol, acetonitrile, cyclohexane), methyl chloride, diethyl ether, polyethylene glycol 400, dimethyl

sulfoxide and chloroform at 10 mM on CGL stability was assessed [44]. The enzyme was dialyzed against 10 mM hydroxylamine for 5 h, to completely dissociate the intrinsic PLP as revealed from the dramatic reduction on the absorbance at 420 nm, to form apo-CGL [33]. The enzyme in potassium phosphate buffer (100 mM, pH 8.0) with each compound was incubated for 1 h at 37°C. The residual enzymatic activity, thermal stability, emitted fluorescence and strength of internal Schiff base were determined as above.

2.12 Curves Fitting and Statistics

Curve fitting was generated using MxPro, Graphpad Prism and Microsoft Excel software. All the experiments were conducted in three biological replicates and the student *t*-test was used to estimate the means and standard deviations.

2.13 Molecular modeling simulation

2.13.1 Homology modeling

A model of CGL enzyme was constructed using x-ray structure of the cystathionine γ -lyase from yeast as template (pdb code: 1N8P) [27] using Modeller program [45]. The sequence identity of both enzymes is 61%, which is high enough to build a reliable model. Another structure of CGL from *Xanthomonas oryzae* [46] is recently resolved with resolution of 2.07 Å. However, the sequence similarity with our target is 50%, which is lower than that of yeast. Since, both structures are very similar (4IXZ and 1N8P, 1.3 Å Root Mean Square Deviation (RMSD) and the secondary structure elements are highly conserved, we decided to go with the eukaryotic model of yeast. To study the enzyme structural and conformational stabilities, the constructed model of CGL was explored with long molecular dynamics simulations. The quality of the initial constructed model was tested by Qmean [47] with a score of 0.718 (Z-score from the distribution of non-redundant high quality structures being only -0.41) which is a highly acceptable model. Also, we evaluated our model using PROSA [48] server which gives overall score of -7.69. This value is close to the value of its template of -9.36.

264

265 **2.13.2 Molecular dynamics simulations**

266 Molecular dynamics (MD) simulations were performed using the GROMACS software (version
267 4.6.5) [49] with the Amber99SB-ILDN force field [50]. The simulations were performed on the model
268 predicted by homology modeling approach as described above. The protein was slightly relaxed using
269 50 steps of steepest descent minimizer. Then after the system was immersed in an explicit water box of
270 TIP3P model [51] which extended at least 11 Å away in each direction from any atom of the complex.
271 The total number of water molecules added to the enzyme was 51814, so the total number of the
272 simulated system is 179838 atoms. Twenty eight sodium ions were added in order to neutralize the
273 negative charge as needed for the Particle Mesh Ewald (PME) [47] calculation of the long-range
274 electrostatic interactions, while cut-off of 10 Å was used for van der Waals (VDW) and short-range
275 electrostatic interactions. Five hundred step of steepest descent minimization were performed to remove
276 the bad contacts with the solvent. All bonds involving hydrogen atoms were constrained by LINCS
277 algorithm [52]. Equilibration of the solvent and ions around the protein with position constraints of the
278 heavy atoms was performed for 200 pico-seconds in the constant Number of particles, Volume, and
279 Temperature (NVT) [53] ensemble and in Constant Number of particles, Pressure and Temperature
280 (NPT) thermodynamic ensemble, respectively. NVT simulations were carried out using the velocity
281 rescaling thermostat (V-rescale) and the NPT using Parrinello-Rahman barostat [54]. MD simulations
282 were performed in the NPT ensemble for 700 ns and the analysis was performed using the last
283 equilibrated 500 ns and Gromacs tools. The Principle Component Analysis (PCA) [55] was performed
284 by diagonalization of the covariance matrix of the C α fluctuations using standard GROMACS tools.

285

286 **2.13.3 Interface dynamics and MMPBSA binding free energy**

The analysis of the contacts between the subunits of CGL on conformations extracted from MD simulations was performed using MDcons. In MDcons analysis, two residues are considered to be in contact if at least two heavy atoms are separated by ≤ 5 Å. The binding free energy was estimated using the end point Molecular Mechanics Poisson-Boltzmann Surface Area (MMPBSA) method [56]. The total free energy (G) in MMPBSA analysis for a CGL (complex (comp), receptor (rec), and ligand (lig)) was determined using Equation (1); the overall change in free energy for complex formation (ΔG_{bind}) for a non-covalent binding event was calculated according to Equation (2).

$$G = G_{\text{polar}} + G_{\text{nonpolar}} + E_{\text{MM}} - TS \quad (1)$$

$$\Delta G_{\text{bind}} = \Delta H - T\Delta S = G_{\text{comp}} - (G_{\text{rec}} + G_{\text{lig}}) \quad (2)$$

The polar solvation energies (G_{polar}) were computed in continuum solvent using generalized born model and ionic force of 0.1 mol. The non-polar terms ($G_{\text{nonpolar}} + \gamma \text{SASA} + \beta$) were estimated using solvent accessible surface areas (SASA in Å²) with typical values for $\gamma = 0.00542$ kcal/mol Å² and $\beta = 0.92$ kcal/mol. The E_{MM} term represented the sum of the electrostatic (Coulombic), VDW (Lennard-Jones), and internal energies (bonds, angles, and dihedrals). The remaining terms represented temperature (T) and solute entropy (S), which can be estimated from normal-mode analysis of energy-minimized structures or quasi-harmonic (QH) modes over stabilized region of MD trajectory. 500 snapshots were selected from the last 100 ns, by keeping the snapshots every 200 ps. Here we neglected the entropy contributions as we are interested in the nature of the interaction and the residues contribution to the total free binding energy rather than the absolute binding free energy. The free energy analysis was carried out using MMPBSA.py script from Amber12 program. The electrostatic calculations were carried out on selected representative structures extracted from MD trajectory using Adaptive Poisson Boltzmann Solver (APBS) program [57].

3. Results

Molecular cloning, expression and Purification of CGL from A. fumigatus

The full length *CGL* gene (*mecB*) (XM_742148.1) of *A. fumigatus* was cloned in pET 28b expression vector in a frame with His-and T7 tags. Recombinant protein was expressed and purified using Ni-resin (Novagen, Madison, WI). The expression and purity of the eluted fractions were analyzed by SDS-PAGE (Fig. 1A) and Western blotting using T7 antibody (Fig. 1B). A prominent protein of about 40 kDa was observed in SDS-PAGE and ensured by western blotting using T7 antibody. The CGL activity of the eluted fractions was determined by the standard assay and the highest active fractions were pooled and concentrated by 30 kDa cut off Amicon membrane. The specific activity of concentrated CGL was 13.8 U/mg. The purified heterologous expressed recombinant CGL from *A. fumigatus* was stored in potassium phosphate buffer (100 mM, pH 7.8) containing 1mM EDTA, 1mM 2-mercaptoethanol at -80°C for further biochemical studies.

Molecular modeling and molecular dynamics simulation of *A. fumigatus* CGL

The molecular model of *A. fumigatus* CGL was constructed using homology modeling approach using the closest yeast CGL homolog as template. Second, MD simulations were conducted for 700 ns and the conformational and structural analyses were performed on the last equilibrated 500 ns. Structurally, CGL consists of two domains sharing other lyases of the overall scaffold of tetrameric assembly (Fig. 2A). The large domain is the PLP-binding, and it comprises seven β -sheets flanked by seven α -helices, while the small domain that adopts the same fold entails four β -sheets surrounded by five α -helices. From the fluorescence emission of CGL for the Tyr and Trp residues; it is worth to dissect their location over the structure. CGL model encompasses two Trp residues in each monomer; the first residue (Trp 157) is anchored in the small domain while the second residue (Trp 254) is located at interface of the four subunits. CGL has nine Tyr_s (60, 62, 85, 116, 122, 195, 214, 292, and 334), the Tyr 116 residue is located in the PLP-binding site and directly involved in pi-stacking with PLP cofactor. Concerning the solvent accessibility, four of these Tyr residues are anchored in the PLP-

binding domain (116, 195, 214 and 292) and the Tyr 122 is inserted at the tetrameric interface while the remaining (60, 62, 85 and 334) are solvent exposed.

To ensure the convergence of our simulations, the root mean square inner product (RMSIP)[58] was estimated for the first 10 eigenvectors extracted from the principle component analysis (PCA) on the two halves of the last equilibrated 500 ns trajectory. RMSIP measures the similarity between the subspaces defined on the basis vectors obtained from the PCA calculations. RMSIP value of 1 means identical space and 0 means completely divergent simulations (Fig. 2B). The estimated RMSIP value of CGL was 0.64, which confirms high similarity between the spaces sampled by the two halves of the equilibrated trajectory. Furthermore, we checked the random diffusion of our system using the cosine content of the first 10 vectors [59]. Indeed, the cosine contents of the first 10 vectors are very low which indicate that the enzyme does not suffer from random diffusion (Fig. 2B). The overall structure undergoes fast stabilization and after few nano-seconds it reaches maximum Root Mean Square Deviation (RMSD) value of 4.5 Å from the initial structure with fluctuation of 0.5 Å. The model has 38% difference from its template so it is expected to undergo conformational relaxation and so deviates from its initial structure (Fig. 2B).

CGL Dynamics

We have analyzed the equilibrated 500 ns of CGL trajectory using the PCA technique to resolve the sample conformational states during the MD simulations. Basically, the enzyme free-energy surface was obtained by the projection of MD conformations into the space of eigenvectors extracted from PCA calculations that allows identifying the equilibrium conformational states. This type of analysis is routinely used in the MD community to resolve the equilibrium states [60-63]. PCA was carried out on the C α atoms, considering the equilibrated part of the MD trajectory. The analysis of the CGL (Fig 3A)

indicated an overall high stability of the tetrameric core and the MD simulations sampled different conformations of the flexible non-structural regions (Fig S1).

Further, the dynamic behavior of the four subunits interface was analyzed using MDcons [64] for analyzing the dynamic features of protein–protein interface complexes. Specifically we have analyzed the tetramer interface by dividing the system into two chains, the first one consists of two subunits (residues from 1 to 824, chain A-B) and the second consists of the other two subunits (residues 825-1648, chain C-D). The residue–residue contact map of different intervals along the MD trajectory (Fig. S2) showing the conserved interaction between the four subunits, with low dynamics behavior. The contact map between the dimmer of subunits AB and CD based on the conformations extracted at different intervals of the MD trajectory were consistent.

MDcons overall conservation scores, C50, C70, and C90, corresponding to the fraction of contacts at 50, 70, and 90% of the analyzed conformations, respectively (Table S1), indicate that the inter-dimer (pair of subunits) contacts are significantly conserved during the MD simulations. Indeed, an average fraction of these contacts was 92% that conserved at least in 50% of the analyzed conformations ($C50 > 0.92$), while 74% of them are conserved in at least 90% of the frames ($C90 \sim 0.75$). Overall, MDcons confirms the results of free energy landscape projected in the essential motion of the CGL enzyme which shows high conformational stability of the protein secondary structure and displays flexibility of its non-structural parts (loops, turns and coils).

MMPBSA Dimmer Binding Free Energy

The dimmer binding free energy was estimated using MMPBSA method in order to analyze the nature of interaction between the subunits and the residual contribution (Fig. S3). The binding free energy without the entropy contribution was estimated to be of -400.0 kcal/mol with error of 6 kcal/mol. The low error of the estimated binding free energy confirms the higher stability of the interacting

interface between the CGL dimmer. In Fig S3, the residual decomposition of the total binding free energy designates the electrostatic nature of the molecular interaction between the dimmer subunits. The major contribution to the total binding free energy is an electrostatic of -1432.0 kcal/mol with less contribution of the hydrophobic interaction of -570.7 kcal/mol. Both types of interactions are favoring the binding of the subunits while the counterpart comes from the solvation of 1695.5 kcal/mol of its polar contribution. However, the non-polar solvation is implicated in the subunits complexation by amounts of -93.0 kcal/mol. The high contribution of the solvation reflects the high polarity of the molecular surface of CGL protein, which needs high energy to displace the water molecules during the binding process.

Focusing on the detailed contribution of the different amino acids, we observed the favored hydrophobic interactions for the binding except for one interaction between His8 and Lys1572 which is unfavorable. These two residues are maintained in this orientation by solvent and strong hydrogen bonds established between His8, Lys1572 and Asp1628 (Fig. 3B). In terms of electrostatic interactions, the major contribution is attributed to residues Arg430, Asp1255, Arg843 and Arg19. The high contributions of these residues in the electrostatic term are compensated by their unfavorable contributions to the desolvation term. For instance residue Arg430 contributes to the electrostatic term by -128.7 kcal/mol, which compensate by +120.9 kcal/mol of the desolvation energy (Fig. 3B).

Effect of different buffers on structural and thermal stability of CGL

The effect of different buffers of various pHs on the thermal stability (melting temperature, T_m) of *A. fumigatus* CGL was evaluated by DSF assay. The thermal dissociation curves of *A. fumigatus* CGL, dissolved in each buffer, were represented in Fig. 4A-D and the shift in melting temperatures (T_m) was shown in Fig. 4E, normalized to potassium phosphate buffer (pH 7.0) as control. In potassium phosphate buffer (pH 7.0) the T_m value of CGL was 73.3°C with no changes at pH 8.0, while up shifting

by $\sim 2^{\circ}\text{C}$ at pH 6.0 was observed, suggesting the acquired slight thermal structural stability at this pH (Fig 4C). Similarly, in MES and Tris-HCl buffers at pH 6.0 the thermal stability of CGL was increased by 2.5 to 3.0°C , with detectable decrease at alkaline pH for both buffers (Fig 4C, E). In sodium potassium phosphate buffer at pH 6.0 the T_m of CGL was upshifted by about 2.4°C compared to negligible increase at pH 7.0 and pH 8.0 (Fig 4B). In contrary, CAPS and sodium bicarbonate buffers had obvious thermal destabilizing effect on CGL structure (Fig 4A). The T_m was strongly decreased by 4.2°C and 5.4°C in CAPS buffer at pH 9.5 and 10.5, respectively and decreased by ~ 3.3 and 2.3°C in sodium bicarbonate buffer at pH 7.2 and 6.4, respectively. The structural thermal stability of CGL in different buffers could be attributed to the chemical composition of dissolving buffer. In order to figure out the experimental observations, the electrostatic calculations were carried out on selected conformations extracted from MD simulations using APBS program (Fig 5). The pH effect was rationalized in terms of the electrostatic interaction, which depends largely on the protonation states of the surface amino acids by the different pH. Eventually, the electrostatic potential of CGL became more negative by increasing the pH (Fig 5). The electrostatic calculations of CGL indicate high correlation of the pH with the electrostatic field generated around the CGL molecule (Fig 5).

To validate the effect of buffer composition on the stability of CGL tertiary structure, the intrinsic fluorescence of its hydrophobic tryptophan residues was measured at 340 nm emission. There is a slight variation on the intensity of emitted fluorescence by CGL at 340 nm in response to various buffers (Fig 6A). The lowest emitted fluorescence for CGL was recorded in potassium phosphate (pH 7.0 and 8.0), MES (pH 6.0), Tris-HCl (pH 8.0) and Phosphate buffered saline, revealing the obvious conformational stability of CGL. In contrary, the emitted fluorescence of CGL in CAPS (pH 9.5 and 10.5), HEPES (pH 7.0) and sodium bicarbonate (pH 7.0) buffers was noticeably increased by about 20-30% than control. This can be explained by the partial unfolding of CGL in these buffers, therefore, exposing the internal hydrophobic core of tryptophan residues to surface polar environment emitting a

higher fluorescence. To explain the experimental increasing on the fluorescence emission at 340 nm, a model of CGL was constructed using homology-modeling approach. The 3D structure of CGL showed that there are two Trp residues per each subunit, four of them at the assembled interface of the subunits and one Trp residue located in the small domain of each subunit (Fig. 6B). There are eight residues of tryptophan per each CGL molecule, four of them buried and four at the subunits interface. We explained the emitted fluorescence is due to the conformational changes of the four Trp residues located at the interface of the subunits to the surface. Also, the relative higher emitted fluorescence of CGL in case of CAPS buffer may be due to the stability of the unfolded state. Indeed, upon unfolding of CGL, the four Trp residues at the interface of the subunits became solvent exposed and stabilized by non-polar solvents like CAPS (Fig. 6 and Supplementary Fig S4).

Since the conversion of active PLP ketoenamine to inactive enolimine is the common effector on activity of PLP-dependent enzymes [35], the tautomeric equilibrium of PLP of CGL in response to buffer composition was assessed at fluorescence excitation wavelengths 330 nm and 410 nm (Fig 7A). Blanks of buffers were used as control and the emitted fluorescence of each buffer is subtracted from the corresponding value of enzyme fluorescence. At 330 nm excitation, the maximum emitted fluorescence at 460 nm was recorded for CGL in sodium potassium phosphate (pH 8.0), MES (pH 7.0) and potassium phosphate buffer (pH 8.0). While at 410 nm excitation the highest emitted fluorescence was observed for CGL dissolved in sodium potassium phosphate (pH 8.0), MES (pH 7.0) and potassium phosphate buffer (pH 8.0), revealing the presence of active form of ketoenamine-PLP in these conditions. For clarification of this assumption raised by the PLP tautomeric equilibrium the colorimetric activity of CGL in each buffer was assessed. Regarding to KP buffer, the enzyme retains 100% of its activity in KP (pH 8.0), Tris-HCl (pH 7.0-8.0), HEPS (pH 6.0), phosphate buffer saline (pH 7.0) and MES (pH 6.0), with a relative decreasing on the CGL activity by 20-35 % for the other buffers

at various pHs (Data not shown). On the other hand, the activity of CGL was decreased by about 50% in CAPS buffer at pH 9.5-10.5 compared to its initial activity in KP buffer at pH 8.0.

The association of co-enzyme PLP with CGL to form holo-CGL was evaluated from the intensity of internal Schiff base at 420 nm (Fig. 7B). From the UV- Spectra, the strength of PLP-Schiff base was relatively dependent on the buffer composition rather than pH range. Among the tested buffers, the highest intensity for CGL was measured in sodium potassium phosphate (pH 6.0), then potassium phosphate buffer (pH 6.0-8.0), and MES (pH 6.0-7.0) with relative decrease in the internal Schiff base among other buffers. Apparently, it could be deduced that buffers with narrow pH range have no obvious negative effect on dissociation of PLP internal Schiff base of CGL. The release of free PLP in reaction medium in response to cleavage of the internal Schiff base was also checked at 390 nm. There is no remarkable variation in absorbance at 390 nm among tested buffers (data not shown), which is consistent with the results of internal aldimine linkage at 420 nm (Fig. 7B). Therefore, based on catalytic activity, we used potassium phosphate buffer at pH 8.0 for further structural and kinetic studies of CGL.

Effect of stabilizing/ destabilizing compounds on CGL conformational stability

The effect of various cosolvents and unfolding agents on stability of the tertiary catalytic structure of CGL was assessed from the colorimetric activity, and thermal stability. The relative activity of CGL in presence of various compounds was assessed. Higher activity of CGL was obtained in presence of PLP (0.4 mM), trehalose, sucrose, glycerol, and ethylene glycol (Fig. 8). A predictable increasing on the activity of CGL upon PLP addition since PLP is the standard co-enzyme, strengthen the internal Schiff base, therefor accelerating the kinetics of substrates γ -elimination to ketobutyrate, cysteine and ammonia. On the other hand, a detectable inhibition of CGL activity was observed in the presence of other denaturants with plausible repression by urea, guanidine hydrochloride (50-60%

reduction) than organic solvents such as isopropanol, diethyl ether, methyl chloride, cyclohexane, acetonitrile, 1,2-propandiol and DMSO (Fig 8). Other compounds such as EDTA, DTT, Tween-20, and Triton X-100, have no effect on CGL activity compared to control condition. To verify the impact of these compounds on the stability of the tertiary folded structure of CGL, the fluorescence emission spectra of intrinsic tryptophan were measured at excitation 295 nm. The spectra of each compound at the same excitation and emission wavelengths was measured as baseline and subtracted from the samples spectra. There is no increase on the intensity of emitted fluorescence of intrinsic tryptophan (at 340 nm) by CGL in presence of cosolvents as sucrose, trehalose, glycerol and ethylene glycol, approving the stabilizing osmolyte/ cosolvent activity to CGL conformational structure. Other organic solvents as ethanol, methanol, cyclohexane, isopropanol, 1,2-propandiol, acetonitrile, diethyl ether and methyl chloride displayed a dramatic increase on the emitted fluorescence at 340 nm (Data not shown) ensuring the unfolding of assembled tetrameric structure of CGL. The plausible increase on the emitted fluorescence at 340 nm was resolved from the molecular modeling simulations. The nature of the hydrophilic surface of CGL stabilized by the polar solvent and destabilized in non-polar solvents. The molecular stability of the CGL molecule regarding to cosolvents, could be strongly explained based on chemical structure of the cosolvents and the enzyme surface. The molecular structure of these compounds was illustrated (Supplementary Fig S4). The negative charged surface of CGL in absence of polar solvent displays high electrostatic repulsion, which induces unfolding of the enzyme while the hydrophobic core is stabilized by the non-polar solvents. Altogether, this drives the thermodynamic equilibrium between the folded/unfolded states to the unfolded one in case of the non-polar solvents. Regarding to the effect of sucrose and trehalose on the stability of CGL, this effect may be related to the polar nature of the molecular surface of CGL (total charge is -28). This was evidenced by the electrostatic calculations performed on equilibrated structure of CGL by MD simulations at different pH range. Actually this decreasing on pH induces the deprotonation of the surface acidic residues (Asp and

Glu), which makes electrostatic potential on the molecular surface of CGL more negative (Fig. 5). Therefore, the structure of CGL could be stabilized *via* polar interaction such as hydrogen bonds and electrostatic interactions with the surface residues mainly Asp and Glu. An obvious relation between the total number of hydrogen bonds between the buffer/ cosolvent and CGL surface residues and the overall conformational stability of CGL. The maximum CGL stability was observed in presence of sucrose and trehalose (maximum hydrogen bonds) while less stability was recorded for the polyethylene glycol (less hydrogen bonds) (Supplementary Fig S4).

The thermal shift in the melting temperatures (T_m) of CGL in response to these compounds was evaluated from the obtained DSF monophasic dissociation curves (Fig 9). The shift in T_m of CGL was normalized to control (without inhibitors). All the tested compounds with various concentrations have a determinable thermal destabilizing effect on CGL conformational structure by decreasing its thermal T_m value. From the melting curves, isopropanol, ethylene glycol, ethanol, 1,2-propandiol, acetonitrile, methyl chloride, methanol and diethyl ether decreases the T_m of CGL by $\sim 4.0^\circ\text{C}$ to 9.0°C . However, glycerol, Tween 20, and EDTA, have no effect on the melting temperature of CGL. In contrary, trehalose and sucrose increases the enzyme melting temperature by 3°C to 4°C . The tertiary structure of CGL was strongly destabilized by GdmCl, the T_m was reduced by ~ 3.9 , 5.4 and 6.0°C at GdmCl concentrations 0.5 , 1.0 and 2.0 M, while in presence of Triton X-100 the T_m was reduced by $\sim 3^\circ\text{C}$ compared to free enzyme (Fig. 9). However, PLP, EDTA, DTT and Tween-20 have no effect on thermal denaturing of CGL. The response of CGL to stabilizing and destabilizing agents was quite different using DSF and steady state fluorescence protocols that were being reasonable since the former technique is temperature dependent.

Conformational Stability of CGL against urea and guanidine hydrochloride denaturation

The stability of CGL structure against urea and GdmCl was evaluated using steady state (Fig 10) and native-PAGE based fluorescence. The enzyme was incubated with the denaturants (0 - 6 M) at 37°C ,

45°C and 55°C, and then the emitted fluorescence was measured. The unfolding curves (Fig 10A, B), clearly shows the sequential increase in fluorescence emission at 340 nm as a function of denaturant concentration and temperature. The $C_{1/2}$ value was expressed by the concentration of denaturant causing 50 % unfolding to enzyme structure [32]. The $C_{1/2}$ values were 2.7, 2.3 and 1.8 M for urea and 2.5, 2.1 and 1.4 M for GdmCl at 37, 45 and 55 °C, respectively. Reasonably, with the higher temperature the $C_{1/2}$ was decreased for synergistic denaturation of CGL. The unfolding of CGL tertiary structure and the progressive increasing in the fluorescence emission upon exposing of the buried four tryptophan residues was checked by the molecular modeling. The instability of CGL structure in urea and GdmCl was explained in terms of the 3D model structure and the solvent structure. CGL structure displayed high negative charged molecule which cannot be stabilized in solvent such as GdmCl because the high electrostatic repulsion between the surface residues (Asp and Glu) and the chloride anions. The stability effect of urea on CGL structure could be similar to isopropanol or methanol as these solvents have a weak stability toward the polar surface of CGL and less hydrogen bonds can be formed with these solvent.

Chemical Modifications of CGL Surface Amino Acids

Chemical triggering of the enzyme catalytic sites to determine its functional state is a well-established strategy [65, 66]. To assess the role of amino acids involved in CGL catalysis and conformational stability, compounds with selective reactivity to certain amino acids were used. Glutamic and aspartic acids carboxyl groups of CGL were modified by EDC; lysine amino groups were protonated by NHS; tryptophan was quenched by chloroform; hydroxyl of serine and threonine was targeted by cyanogen bromide. From the activity (Fig 11), a slight inhibition in CGL activity was detected upon using EDC (10-40 mM) and minor increase in its activity by NHS at 10 mM was observed, suggesting a little implication of glutamic and aspartic acids in catalysis process. On the other hand, a significant reduction

in CGL activity was observed at higher concentrations of CNBr (23% reduction at 40 mM) (Fig 11), suggesting an interaction with surface hydroxyl containing amino acids. Also, the activity of CGL was decreased sequentially as the concentration of chloroform increases losing 50% of its initial activity at 0.4% (Fig 11). The inhibition of CGL activity by chloroform implies the oxidation of subunit interface tryptophan residues causing denaturation and unfolding. The conformational stability, unfolding/folding structure of CGL was visualized on SDS-PAGE based on fluorescence emission of intrinsic tryptophan when oxidized by chloroform. No significant effect of EDC, NHS or CNBr on induction of the hydrophobic core to be exposed to the surface, as consistent with CBB staining of the same gel (Fig 11 B).

The effect of the CNBr on the surface residues that is related to the CGL activity was explained in terms of the 3D model. In the constructed model there are four Ser (91, 93, 210 and 343) and two Thr (190 and 212), which are located in the active site and directly participated in the PLP binding domain (Fig. 12). So, the inhibitory effect of CNBr on activity of CGL could be ascribed to the disturbed direct interaction with the hydroxyl group of Ser and Thr through direct bromination causing changes in the conformation of PLP in CGL active site. From the colorimetric activity and fluorescence emission, it was found that CNBr has a slight effect on the intensity of internal Schiff base, which indicates that PLP is still bound to CGL.

Discussion

In this study we have investigated the conformational and catalytic stability of the recombinant CGL from *A. fumigatus*. The conformational and catalytic stability of fungal CGL in response to various buffers, destabilizing and stabilizing agents have been poorly characterized, unlike the extensive studies on human homolog, regarding to their role in the endogenous H₂S balance [6, 17]. Thus, we hypothesized that analysis of the catalytic and structural characterization of fungal CGL will lay the

574 foundation for its therapeutical exploitations in cardiovascular diseases. Detailed mechanistic study of
575 fungal CGL is also of a prominent importance for its crucial implication in sulfur metabolism,
576 methionine and cysteine cycle in addition to its promising applications in cystathionine related diseases.
577 The CGL encoding gene (*mecB*) from *A. fumigatus* was cloned and expressed in *E. coli* BL21. The
578 activity of the expressed recombinant *A. fumigatus* CGL was 13.8 U/mg compared to the recombinant
579 CGL from human that had specific activity of 2.5 U/mg [5] and the wild *Lactococcus lactis* CGL that
580 has specific activity of 64.7 U/mg.

581 Microbial enzymes are currently approved as universal drugs against cancer, neurological and
582 cardiovascular disorders as reviewed by El-Sayed, [67-71]. However, the conformational instability,
583 premature inactivation and structural antigenicity of the PLP-enzymes are the main practical limitation
584 for their applied therapeutic uses [72-74]. In particular, the PLP-dependent enzymes are the most
585 sensitive ones for physiochemical conditions and compounds that cause rapid loss of their PLP-
586 coenzyme, leading to a dramatic suppression in their activities [75-78]. Thus, the conformational
587 stability of *A. fumigatus* CGL in response to buffer structure, pH and chemical compounds was
588 investigated.

589 The effect of buffer composition and pH on the structural thermal stability of *A. fumigatus* CGL
590 was assessed from the T_m values of DSF assay. The shift in melting temperature of CGL in response to
591 buffer type and pH was evaluated, normalized to potassium phosphate buffer (100 mM, pH 8.0) as
592 control. The stability of CGL was dramatically decreased with the higher temperature that accompanied
593 with decrease in Gibbs free energy of unfolding ΔG_u [79, 80]. At melting temperature (T_m), the ΔG_u is
594 close to zero that is an equilibrium between folded and unfolded state of the enzyme [31, 81]. A slight
595 positive thermal shift stability of CGL by MES buffer at pH 6.0 was consistent with those reported for
596 citrate synthase [31]. Thus, we can deduced that CGL conformation is a protonation dependent as
597 frequently hypothesized [82], where the enthalpy of denaturation is a pH dependent. On the other hand,

down shifting in CGL thermal stability might be due to either buffer protonation by heating causing enzyme denaturation or changing in pH of the buffer upon heating [83]. Similarly, the buffer ionization upon heating was hypothesized for α -chemotrypsin [84] and hexokinase [85]. The stability of the enzymes was strongly dependent on orientation and thermodynamics of the amino acids constituting the α -helix domains [86]. The observed decrease in CGL structural stability by CAPS and Na-bicarbonate buffer might be due to dual action of pH and high protonation and autoxidation by heating, while the thermal stability of CGL by PBS, MES and KP buffers at pH 6.0 could be attributed to scavenging of hydroxyl free radicals of water upon heating [87]. However, our molecular modeling approaches based on model structure of CGL followed by molecular dynamics simulations enhanced the hypothesis that the major effect of the structural stability of CGL is due to the pH changes. The experimental results concerning to catalytic and structural stability was progressively explained regarding to molecular modeling and dynamics. Indeed, the electrostatic calculations on the explored CGL structure using MD simulations showed high dependence on the pH changes. The structure of the fungal CGL is surrounded by negatively charged patch of amino acids in sandwich-like shape (Fig 13). The structural stability of CGL required high interaction with polar solvents that attenuates the electrostatic repulsion between the surface amino acids.

The effect of buffer type and pH on the stability of the tertiary structure of CGL was assessed from the intensity of emitted intrinsic fluorescence at 340 nm and 500 nm upon excitation at 295 nm. The intrinsic fluorescence emission of tryptophan was a reliable approach to assess the folding/unfolding and structural stability of enzymes [35, 75]. Upon enzyme partial unfolding the hydrophobic core of the aromatic amino acids tryptophan are exposed to the surface, and subsequently the emitted fluorescence at 340 nm was increased. Among the tested buffers, CGL dissolved in CAPS buffer (pH 9.5 and 10.5) and sodium bicarbonate (pH 7.0) gave a relatively higher emitted fluorescence at 340 nm, suggesting unfolding of the CGL tertiary structure, thus exposing the buried tryptophan residues to polar

environment [34]. The higher emitted fluorescence of CGL in CAPS buffer at pH 9.5 and 10.5 is confirmed by the structural model where high electrostatic repulsion between the surface amino acids and higher negative pH of the buffers, stabilizing the unfolded state of CGL. The hydrophobic nature of CAPS lacks the screening effect of the electrostatic forces and hence does not induce any stability of CGL. The slight variation in the emitted fluorescence at 340 nm in the presence of other buffers suggest a negligible negative effect on the enzyme tertiary structure as revealed from the balance on the number of exposed aromatic amino acids to surface environment.

Proton transfer from buffer to the enzyme is the main cause of enzyme allosteric or catalytic changes, accompanied with its partial denaturation [88]. In this regard, tautomeric PLP equilibrium is considered one of the main determinant parameters upon amino acid modification and physicochemical effectors on CGL catalysis [35, 36, 89, 90]. The PLP tautomeric equilibrium (ketoenamine and enolimine) of CGL in response to buffer structure and pH was evaluated based on emitted fluorescence at 510 nm and 460 nm, respectively. The stable ketoenamine residues based on the emitted fluorescence was determined for CGL dissolved in sodium potassium phosphate, potassium phosphate buffer (pH 8.0) and MES (pH 7.0). For verification of the effect of buffer type on CGL catalysis, the colorimetric activity was determined. The remaining activity of CGL dissolved in KP, Tris-HCl, HEPS, PBS and MES buffer (pH 6.5-7.0) was similar and fluorometrically homogenous to PLP tautomeric equilibrium analyses.

Association and dissociation of the internal aldimine linkage of PLP is the main determinant factor for catalysis of PLP-dependent enzymes as L-methioninase [33, 67, 69], human CGL [7], decarboxylase [77], and O-acetylserine sulfhydrylase [34, 75]. The intensity of internal PLP Schiff base was assessed in response to buffer composition and pH. The highest intensity of CGL internal aldimine linkage at 420 nm was reported for CGL dissolved in NaKP, then KP and MES buffers compared to

645 other buffers ensuring the positive effect of KP, NaKP and MES buffers on the tertiary structure and
646 catalytic processes of CGL.

647 The effect of various cosolvents and unfolding inducers on stability of the tertiary structure of
648 CGL was assessed from the colorimetric activity, fluorometric assay and thermal stability. The activity
649 of CGL was maximally increased in presence of PLP, trehalose, sucrose, glycerol and ethylene glycol.
650 The increasing of CGL activity by PLP coenzyme addition can be attributed to the formed internal
651 Schiff base and therefore, accelerating the kinetics of γ -elimination of products. The catalytic activation
652 of CGL by trehalose, sucrose, glycerol and polyethylene glycol could be explained by increasing the
653 accessibility of substrate to bind to CGL active sites, since these compounds were repeatedly used as
654 cosolvents [91]. It was hypothesized that these compounds increase the preferential hydration of CGL
655 backbone and therefor stabilizing the enzyme folded active state [44, 91]. Trehalose is a universal
656 stabilizer with inert reactivity with protein surface, counteracting the deleterious environmental effect
657 on proteins as revealed from the increasing of the positive transition melting temperature (ΔT_m) of
658 RNase [91]. Mechanistically these compounds can stabilize the CGL by decreasing its heat denaturing
659 capacity *via* inducing preferential hydration of its backbone. The experimental results of the cosolvents
660 (trehalose, sucrose and polyethylene glycol) on the thermal stability of CGL were rationalized using
661 molecular modeling approaches. Trehalose and sucrose solvent are multi-hydrogen bond donors that can
662 stabilize CGL structure. The inhibitory effect of polar solvents on CGL activity could be attributed to
663 dehydration of enzyme backbone and/or interacting with the hydrophobic residues causing net
664 destabilizing/ unfolding of the enzyme tertiary structure [84, 92]. Unfolding/ folding equilibrium of
665 enzymes as function of GdmCl concentration were studied for other enzymes as keto acyl-ACP
666 reductase [93], L-asparaginase [74, 94], and hexokinase [95]. In this regard, McClure and Cook, [34],
667 Bettati et al. [75] attributed the increase in the emitted fluorescence at 340 nm in response to GdmCl
668 concentrations to enzyme denaturation and therefor, exposing the buried four tryptophan residues to the

aqueous environment. The experimental effect of the GdmCl can be explained by the electrostatic model with regard to its charge. GdmCl has a negative charge of -1 which induces high electrostatic repulsion with the negatively charged surface residues of CGL which induces the unfolding of the assembled subunits. The unfolding of CGL tetrameric structure by urea and GdmCl as a function of temperature was evaluated using fluorimetry based emitted fluorescence. With the denaturant concentration and reaction temperature, the fluorescence emission at 340 nm was increased sequentially due to the exposing of buried tryptophan and tyrosine residues of the hydrophobic core to the polar environment [32, 86, 96]. The unfolding of CGL was strongly dependent on the denaturant concentrations and reaction temperature.

The catalytic and conformational stability of CGL was evaluated by specific triggering of certain surface amino acid residues [65]. A slight inhibition of CGL activity was detected by EDC, unlike the minor increase in activity by NHS, suggesting a minor implication of glutamic and aspartic acids in the catalysis process. A dramatic reduction in CGL activity was observed by CNBr and chloroform, suggesting the oxidation of the surface hydroxyl amino acids and tryptophan, respectively. The intensity of emitted tryptophan fluorescence by CGL towards these compounds was relatively similar, suggesting a negligible effect on CGL structure and orientation of tryptophan residues, which is consistent with the colorimetric activity. The effect of the CNBr on the surface residues, which is related to the CGL activity, can be explained in terms of the constructed structural 3D model. In our model there are two Ser residues (91, 93, 210 and 343) and Thr residues (190 and 212) in the PLP-binding site. These residues have direct interactions through many hydrogen bonds with the co-enzyme. These interactions could have an important catalytic role *via* the tuning of PLP conformations to be appropriate for the catalysis. Thus any modifications of the hydroxyl group of these residues, which are exposed to solvent, can clearly impact the PLP orientations.

692 In conclusion, this work was motivated to assess the conformational and catalytic stability of the
693 recombinant *A. fumigatus* CGL coupled with molecular modeling simulations in response to various
694 biochemical factors. The structural stability of the recombinant CGL towards the studied effectors was
695 evaluated from the thermal shifting in melting temperature, unfolding of enzyme through exposing the
696 intrinsic hydrophobic core to polar environment, as measured from the steady state-based emitted
697 fluorescence. The colorimetric activity and strength of the internal Schiff base and free PLP was
698 measured as well. CGL is the key enzyme on sulfur assimilation and antioxidants synthesis in fungi as
699 well as endogenous generating H₂S as signaling molecule in human and plant cells. The catalytic
700 stability of CGL is correlated with the intensity of internal Schiff base by the bounded PLP moieties.
701 The enzyme displayed a plausible thermal stability in potassium phosphate buffer followed by MES,
702 PBS and NaKP buffer at 100 mM of pH 7.0, in contrary to the lower stability in CAPS and Na-
703 bicarbonate buffer. The conformational stability of CGL was decreased accordingly with the function of
704 temperature increasing that could be attributed to the buffer protonation thus ionization to enzyme
705 functional domains. Based on the intrinsic emitted fluorescence, CGL dissolved in CAPS buffer (pH
706 9.5) and Na-bicarbonate (pH 7.0) exhibited a relatively higher emitted fluorescence, suggesting the
707 unfolding of the enzyme structure, thus exposing the buried tryptophan residues to polar environment.
708 The enzyme in Na-KP buffer, KP buffer and MES (pH 7.0) had a stable ketoenamine residues based on
709 the emitted fluorescence, suggesting the little protonation pumping by these buffers. Also, in these
710 buffers the enzyme had a stable internal aldimine linkage more than other buffers. The stability of CGL
711 conformational structure was significantly increased by trehalose and glycerol as cosolvents, in contrary
712 to significant denaturing and unfolding by GdmCl and urea with the function of temperature and
713 concentration. Our molecular modeling simulations were necessary to explain the various experimental
714 results and correlated them with the 3D structure of CGL. Based on our model we could establish the
715 electrostatic model which explained the effect of the different buffers like CAPS and cosolvents like

trehalose and sucrose. We would like to emphasize that the promising current biochemical and conformational stability and robustness of fungal CGL further warrants its exploitation for therapeutic use in cardiovascular and neurological disorders, comparing to human enzyme.

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Figure legends

Fig.1: Expression and purification of *A. fumigatus* CGL. The coding sequence of *A. fumigatus* CGL was cloned in pET28b in frame with T7 and His-tags, expressed and purified using His-Resin. The crude

982 extract and the eluted fractions were tested by Coomassie staining (A) and Western blotting using anti
 983 T7 tag antibody (B).

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 985 **Fig. 2:** Secondary structure of the tetrameric complex of CGL (pdb:1N8P) and the monomeric subunit
 986 (A). PLP is shown in green balls. Detailed view of the most important amino acids involved in the
 987 interaction of PLP in the active site is shown in sticks.

989 **Fig. 3:** Differential Scanning Fluorometry of *A. fumigatus* CGL in various buffers. CGL was dissolved
 990 in CAPS, HEPES, and sodium bicarbonate (A), Tris-HCl, and sodium potassium phosphate (B),
 991 potassium phosphate and MES (C), phosphate buffered saline and MQ water (D). Overall shift in the
 992 melting temperature (T_m) of CGL in the presence of different buffers (E). Melting temperature (T_m)
 993 was calculated (arbitrary fluorescence units (AFU)/ dT) as a function of temperature increments.

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1008 **Fig. 4:** The secondary structure of the tetrameric complex of CGL and ± 5 kT/e electrostatic potential
1009 isocontours around the tetramer complex extracted at 700ns at different pH range. A is the secondary
1010 structure of tetrameric complex. B, C and D are the electrostatic potentials at pH 6.0, 9.5 and 10.5,
1011 respectively. The electrostatic calculation was performed using APBS program (Materials and Method).
1012 *Note:* The isocontours of the D was generated with ± 10 kt/e for sake of clarity.

Fig. 5: The intensity of emitted fluorescence at 340 nm of *A. fumigatus* CGL at 295 nm excitation using the same buffers as baseline at the same condition (A). The secondary tetrameric structure of *A. fumigatus* CGL constructed using the yeast structure (pdb 1N8P) as template (B). In the left panel Trp residues are shown in red spheres, while in the right panel a magnified image of the four Trp located at the subunits interface.

Fig. 6: The fluorescence emission and UV-spectra of CGL in response to different buffers. The intensity of emitted fluorescence at 460 nm and 510 nm was shown under 330 nm and 410 nm excitations, respectively, to assess the tautomeric equilibrium of PLP ketoenamine and enolimine (A). The UV absorbance at 420 nm to determine the intensity of internal PLP aldimine linkage of CGL was shown (B).

Fig. 7: Relative activity of *A. fumigatus* CGL in response to various cosolvents and denaturants. The standard reaction mixture of CGL containing cystathionine was supplemented with each compound, incubated at 37 C, and then the residual activity was measured as described in Materials and Methods.

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1060 **Fig. 8:** Differential Scanning Fluorimetry (DSF) of CGL in response to various stabilizing and
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Fig. 9: Conformational Stability of CGL against urea (A) and guanidine hydrochloride (GdmCl) (B). The enzyme was incubated with different concentrations of urea and GdmCl for 20 min at 37, 45 and 55°C. The intrinsic fluorescence emission of CGL was measured at 340 nm upon 295 nm excitation.

Temp. (°C)	$C_{1/2}$ of urea (M)	$C_{1/2}$ of Guanidine chloride (M)
37	2.7	2.5
45	2.3	2.1
55	1.8	1.4

$C_{1/2}$ values are the concentration of denaturants causing half-unfolding to CGL tertiary structure

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1077 **Fig. 10:** Activity of *A. fumigatus* CGL (A), and native-PAGE based on fluorescence visualization (B,
1078 upper) of the enzyme hydrophobic core and CBB gel stained (B, lower) in response to various amino
1079 acids targeting inhibitors. For assessment of the CGL unfolding, the gel was soaked in chloroform for
1080 10 min and then illuminated by UV- Transilluminator at 305 nm.

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1089 **Fig. 11:** Structural model of *A. fumigatus* CGL with atomic details of PLP binding site of chain A. PLP
1090 is shown in CPK representation and the surrounding residues are shown in sticks with focusing on Ser
1091 and Thr amino acids. The hydrogen bonds involving Ser and Thr are shown by dashed line.

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