Structurally disordered C-terminal residues of GTP cyclohydrolase II are essential for its enzymatic activity

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ABSTRACT
GTP cyclohydrolase II (GCHII) is one of the rate limiting enzymes in riboflavin biosynthesis pathway and is shown to be a potential drug target for most of the pathogens. Previous biochemical and structural studies have identified the active site residues and elucidated the steps involved in the catalytic mechanism of GCHII. However, the last ~20–25 C-terminal residues of GCHII remains unstructured in all the crystal structures determined to date and their role in the catalytic activity, if any, remains elusive. Therefore, to understand the role of these unstructured C-terminal residues, a series of C-terminal deletion mutants of GCHII from Helicobacter pylori (hGCHII) were generated and their catalytic activity was compared with its wild-type. Surprisingly, none of the C-terminal deletion mutants shows any enzymatic activity indicating that these are essential for GCHII function. To get additional insights for such loss of activity, homology models of full-length and deletion mutants of hGCHII in complex with GTP, Mg\(^{2+}\), and Zn\(^{2+}\) were generated and subjected to molecular dynamics simulation studies. The simulation studies show that a conserved histidine at 190\(^{th}\) position from the unstructured C-terminal region of hGCHII interacts with \(\alpha\)-phosphate of GTP. We propose that His-190 may play a role in the hydrolysis of pyrophosphate from GTP and in releasing the product, DARP. In summary, we demonstrate that the unstructured C-terminal residues of GCHII are important for its enzymatic activity and must be considered during rational drug designing.

Abbreviations: DARP: 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5'-phosphate; DTT: Dithiothreitol; GCHII: GTP cyclohydrolase II from Helicobacter pylori; GCHII: GTP cyclohydrolase II; GMP: Guanosine monophosphate; GMPCPP: Guanosine-5’-[(\(\alpha,\beta\))-methylene]-triphosphate; GTP: Guanosine triphosphate; PPi: Pyrophosphate

Introduction
Antibiotic resistance is a serious public health problem killing an estimated 700,000 people every year worldwide as most of the bacteria are becoming resistant to conventional antibiotics. In 2017, the World Health Organization (WHO) has published a list of drug-resistant bacteria that are greatest threat to human health (Dang & Graham, 2017; Willyard, 2017). Such an increase in bacteria, that are resistant to existing drugs, stressed the necessity to explore for new drug targets. The enzymes of riboflavin biosynthesis pathway; riboflavin biosynthesis pathway; Helicobacter pylori
The role of manganese ion in GCHII has been proposed (Kaiser et al., 2002; Ritz et al., 2001; Spoonamore et al., 2006). In the proposed catalytic mechanism, the first step is the release of pyrophosphate by nucleophilic attack on the $\alpha$-phosphorous of GTP and formation of a covalent intermediate of guanylated enzyme. The guanidinium group of Arg-128, located between the $\alpha$- and $\beta$-phosphate groups of GTP, is speculated to act as a nucleophile and forms a covalent adduct with the enzyme (Ren et al., 2005). A magnesium ion, positioned between the $\alpha$- and $\beta$-phosphonate groups of GTP, neutralizes the negatively charged groups of nucleotide. The second step is the ring opening of the guanine group of GTP. The $Zn^{2+}$ coordinates with three cysteine residues Cys-55, Cys-66 and Cys-68 and a water molecule. This water molecule is polarized by $Zn^{2+}$ causing an increase in its acidity and acts as a nucleophile. Since this water molecule is also located close to guanine imidazole moiety, it is speculated to carry out the nucleophilic attack on the C-8 atom of GTP. The hydroxyl group of Tyr-105 may act as a proton donor to N-7 atom of GTP. The rearrangement of substrate after water addition, lets the ring opening and produces a formamide derivative intermediate. Further round of water addition from zinc ion releases the formate. Finally, the release of product, DARP from the enzyme occurs by the hydrolysis of nucleotide from Arg-128 (Ren et al., 2005; Spoonamore et al., 2006; Spoonamore & Bandarian, 2008). Thus, both $Zn^{2+}$ and $Mg^{2+}$ are essential for the catalytic activity of GCHII (Ren et al., 2005).

The second crystal structure of GCHII has been solved from *M. tuberculosis* (mGCHII) as part of a bifunctional enzyme with 3,4-dihydroxy-2-butane 4-phosphate synthase (DHBPS) and GCHII at its N- and C-terminal region, respectively (Singh et al., 2013). The overall structure of mGCHII is similar to eGCHII suggesting a similar mechanism of catalysis. Importantly, both eGCHII and mGCHII crystal structures show an intrinsically bound $Zn^{2+}$ which is coordinated with three catalytic cysteines and a water molecule. The crystal structure of GCHII from *H. pylori* (hGCHII) is recently reported (Yadav & Karthikeyan, 2015). While the overall crystal structure of hGCHII is similar to mGCHII and eGCHII, it is devoid of intrinsically bound $Zn^{2+}$ with its coordinating cysteines found in oxidized form, thus exhibiting the inactive state of hGCHII. It has been speculated that the hGCHII might show a redox dependent catalysis and it can switch its activity from inactive (oxidized) to active (reduced) state depending upon the redox environment (Yadav & Karthikeyan, 2015).

In this study, we have investigated the functional contribution of disordered C-terminal residues of hGCHII and the results reveal that the C-terminal residues are essential for the catalytic activity of hGCHII. In addition, we show that the C-terminal residues of GCHII in *E. coli* and *S. typhimurium* are also essential for their catalytic activity. Furthermore, molecular dynamics simulations of modeled full-length structure of hGCHII in complex with GTP, $Mg^{2+}$ and $Zn^{2+}$ display a stable interaction between His-190 of unstructured C-terminal region and substrate, GTP. We propose that His-190, being a conserved residue at this position, may play a role in the hydrolysis of pyrophosphate from GTP and possibly in releasing the product, DARP.

### Material and methods

#### Cloning of full-length and C-terminal deletion mutants of GCHII

In bacteria, the GCHII enzyme is encoded by the gene *ribA*. To clone the full-length *ribA* in a bacterial expression vector, the gene was amplified by polymerase chain reaction (PCR) using the genomic DNA of respective bacterium with forward and reverse primers (Table 1). The PCR amplified product was evaluated in an agarose gel and subsequently extracted and digested with *NdeI* and *XhoI* restriction enzymes (Fermentas, USA). The digested PCR product was further purified by using the PCR cleanup kit (Qiagen, Germany) and then ligated into pET28b vector, which was pre-digested with *NdeI* and *XhoI* restriction enzymes, followed by its transformation in DH5α cells. The positive clone with the desired DNA insert was confirmed by automated DNA sequencing. To generate the C-terminal deletion mutant

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*Figure 1.* Reaction catalyzed by GCHII enzyme in the presence of $Mg^{2+}$ and $Zn^{2+}$. The major product, DARP shows absorbance at 310 nm.
clone, a stop codon (TAA) was introduced into the reverse primer such that the translation would terminate at the desired amino acid position. Using the modified reverse primer (Table 1), each C-terminal deletion mutant gene was amplified by PCR using respective full-length clone as a template and cloned into pET28b vector. Using the above

Table 1. Primers used for the amplification of ribA genes and their C-terminal mutants.

| S. no. | Clone/protein name | Species    | Residues covered    | Forward (restriction enzyme sites are underlined) | Reverse (restriction enzyme sites are underlined) |
|-------|--------------------|------------|---------------------|---------------------------------------------------|-------------------------------------------------|
| 1.    | hribA/ribHII       | H. pylori  | 1–192 (wild-type)  | Forward: 5’ ATA CCT AGA CAT ATG TTA GGA GCT 3'  |
|       |                    |            |                     | Reverse: 5’ ATT ATA TTG GAG TCA GTA CCA GTC 3’  |
| 2.    | hribA188/ribHII188 | H. pylori  | 1–188 (deletion mutant)  | Forward: 5’ ATA CCT AGA CAT ATG TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATA TTG GAG TCA GTA CCA GTC 3’  |
| 3.    | hribA177/ribHII177 | H. pylori  | 1–177 (deletion mutant)  | Forward: 5’ ATA CCT AGA CAT ATG TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATA TTG GAG TCA GTA CCA GTC 3’  |
| 4.    | hribA177/ribHII171 | H. pylori  | 1–171 (deletion mutant)  | Forward: 5’ ATA CCT AGA CAT ATG TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATA TTG GAG TCA GTA CCA GTC 3’  |
| 5.    | eribA/ribEII       | E. coli    | 1–196 (wild-type)  | Forward: 5’ GAG AGT CAT ATG TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATT TTA GGA GCT 3’  |
| 6.    | enibA/ribEII       | E. coli    | 1–196 (wild-type)  | Forward: 5’ GAG AGT CAT ATG TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATT TTA GGA GCT 3’  |
| 7.    | enibA190/ribEII190 | E. coli    | 1–196 (wild-type)  | Forward: 5’ GAG AGT CAT ATG TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATT TTA GGA GCT 3’  |
| 8.    | eribA179/ribEII179 | E. coli    | 1–179 (deletion mutant)  | Forward: 5’ GAG AGT CAT ATG TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATT TTA GGA GCT 3’  |
| 9.    | snibA/ribSII       | S. typhimurium | 1–196 (wild-type)  | Forward: 5’ ATT ATT TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATT TTA GGA GCT 3’  |
| 10.   | snibA190/ribSII190 | S. typhimurium | 1–190 (deletion mutant)  | Forward: 5’ ATT ATT TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATT TTA GGA GCT 3’  |
| 11.   | snibA179/ribSII179 | S. typhimurium | 1–179 (deletion mutant)  | Forward: 5’ ATT ATT TTA GGA GCT 3’  |
|       |                    |            |                     | Reverse: 5’ ATT ATT TTA GGA GCT 3’  |
| 12.   | snibA179/ribSII173 | S. typhimurium | 1–173 (deletion mutant)  | Forward: 5’ ATT ATT TTA GGA GCT 3’  |

Figure 2. Design and construction of GCHII mutants. Multiple sequence alignment of hGCHII (192 aa), sGCHII (196 aa), and eGCHII (196 aa). Identical residues are shown in white with a red background, whereas similar residues are shown in red. The secondary structure of hGCHII as predicted by PSIPRED server is shown in cartoon form and mapped on to the multiple sequence alignment. In the cartoon, the helix, strand and random (coil) structures are represented as cylinder (magenta), arrow (yellow) and line (black), respectively. The sequence number for hGCHII is marked on top of the alignment. The C-terminal disordered region of last 20 residues of hGCHII is shown in box with dashed lines. The vertical blue lines indicate the design of eGCHII and sGCHII deletion mutants with respect to hGCHII.
Expression and purification of GCHII and its mutants

For the expression of GCHII and its mutant proteins, a plasmid carrying a gene of interest was transformed into BL21 (DE3) cells and streaked on a Luria-Bertani Broth (LB) agar-kanamycin (30 μg/mL) plate. Subsequently, the plate was incubated at 37°C overnight for the growth of colonies. A single colony from the plate was picked and used to inoculate a 10 mL LB media supplemented with kanamycin and allowed to grow as seed culture at 37°C overnight for the growth of colonies. The cell culture at 600 nm (OD600nm) reached a value of 1.0, and allowed to grow for 2 h at 37°C at 200 RPM. A secondary culture of 500 mL of LB media supplemented with kanamycin was inoculated with 1% (v/v) of overnight grown seed culture and allowed to grow for 2 h at 37°C at 200 RPM, after which the temperature was reduced to 25°C. Once the optical density of the cell culture at 600 nm (OD600nm) reached a value of ~0.6–0.8, the protein expression was induced by the addition of 0.5 mM IPTG and the cell culture was further grown for 12 h at 25°C. The cells were harvested by centrifuging the culture at 6000 x g for 10 min at 4°C. After discarding the supernatant, the cell pellet was resuspended in lysis buffer containing 50 mM Tris–base pH 8.0, 150 mM NaCl, 1 mM DTT, and a protease inhibitors cocktail (1 tablet/500 mL culture) (Roche, USA). The resuspended cells were lysed by sonication (Sonics, USA) for 15 min with 30 s ON/OFF pulse at 20% amplitude. The cell lysate was centrifuged at 4°C for 45 min at 15,000 x g. The supernatant was passed through Ni–NTA column (Qiagen, Germany) which was pre-equilibrated with 50 mM Tris–base pH 8.0, 150 mM NaCl, and 1 mM DTT. The column was washed with 25-column volume of wash buffer-1 (50 mM Tris–base pH 8.0, 150 mM NaCl, and 1 mM DTT) followed by the 25-column volume of wash buffer-2 (50 mM Tris–base pH 8.0, 150 mM NaCl, and 30 mM imidazole). Finally, the Ni–NTA bound protein was eluted with elution buffer (50 mM Tris–base pH 8.0, 150 mM NaCl, 1 mM DTT, and 200 mM imidazole). The eluted protein was dialyzed against 25 mM Tris-base pH 8.0, 100 mM NaCl, and 2 mM DTT at 4°C with two buffer changes at an interval of 7–8 h. For further purification, the protein was subjected to size exclusion chromatography using the Sephacryl S-200 column (GE Healthcare, USA). The purified protein was concentrated using a 10 kDa cut off Amicon concentrator (Millipore, USA) and Bradford method (Bradford, 1976) was used for the estimation of protein concentration. The purity of protein was checked on 15% SDS-PAGE. All the GCHII and mutant proteins were expressed and purified using the above method.

Enzyme activity of GCHII and its mutants

The enzymatic activity of GCHII, and its mutants was measured spectrophotometrically as described previously (Lehmann et al., 2009). Briefly, a reaction mixture of 500 μL consisting of 50 mM Tris-base, pH 8.0, 100 mM NaCl, 2 mM DTT, 10 mM MgCl2, and 50 μM GTP was taken and the reaction was started by adding the fixed amount (50 μg) of GCHII or mutant protein. Although, Zn2+ was required for the catalytic activity of GCHII, addition of external Zn2+ to the reaction did not have any effect on the activity of GCHII as it was intrinsically bound to the enzyme (Yadav & Karthikeyan, 2015). Therefore, the enzymatic activity of GCHII and their mutants was carried out in the absence of externally added Zn2+. The formation of product, DARP was monitored by measuring the time-course absorbance at 310 nm for 5 min using UV–Visual spectrophotometer (Perkin Elmer Lambda 25, USA). The data were plotted using Origin 8.5 software.

Modeling of full-length hGCHII in complex with its substrate

The full-length hGCHII structure (residues 1–192) was modeled using the I-TASSER server by providing E. coli GCHII structure (eGCHII; PDB ID: 2BZ0) as a template (Yang et al., 2015). For the current study, the full-length ribA from E. coli (eribA; 591 base pairs), and S. typhimurium (sribA; 579 base pairs) were cloned into pET28b vector (Yadav & Karthikeyan, 2015). For the current study, the full-length ribA from H. pylori (hribA; 579 base pairs) was cloned in pET28b vector (Yadav & Karthikeyan, 2015). For the current study, the full-length ribA from H. pylori (hribA188, hribA177, and hribA171), E. coli (eribA190, eribA179, and eribA175), and S. typhimurium (sribA190, sribA179, and sribA173) were also cloned in pET28b vectors (See Table 1 for the details).
The monomeric form of a predicted model of hGCHII was then converted into dimer by superimposing two monomers on the E. coli GCHII dimer structure (PDB ID: 2BZ0). The structure of hGCHII bound with guanosine 5’-(α,β-methylene) triphosphate (GMPCPP), Zn²⁺, and Mg²⁺ was created by transferring their coordinates from eGCHII structure to hGCHII dimer. The GMPCPP in the model was then converted to GTP by replacing a carbon atom of α/β-phosphonate group with an oxygen atom. The C-terminal deletion mutants of hGCHII-GTP complexes were prepared by removing last 4-, 15-, and 21-residues from the modeled full-length hGCHII-GTP complex structure.

**Molecular dynamics simulations of hGCHII and its deletion mutants**

The hGCHII-GTP complexes (hGCHII-GTP, hGCHII₁⁸⁸-GTP, hGCHII₁⁷⁷-GTP, and hGCHII₁⁷¹-GTP) were prepared for MD simulations using the LEaP module of AMBER14 (Pearlman et al., 1995). Initially, hGCHII-GTP was prepared with the ff14SB force field (Maier et al., 2015) which assigned topology parameters and added required hydrogen atoms to the residues. Residues Cys-55, Cys-66, and Cys-68, coordinating the Zn²⁺, were designated as deprotonated. Force field parameters and charges for GTP were obtained from the amber parameter database maintained at the University of Manchester, UK (Meagher et al., 2003). The Zn²⁺ and Mg²⁺ ions present in the active site were assigned a compromise (CM) set of ion parameters (Li et al., 2013). The hGCHII-GTP complex was then surrounded by a truncated octahedral box of explicit water molecules defined with TIP3P water model (Jorgensen et al., 1983). The edge of the box was kept at 8.5 Å from the protein. The requisite counter ions were then added to neutralize the system. In the end, the LEaP module created a topology file containing force field parameters for protein, GTP, ions, and water molecules and created a coordinate file containing structural information for the full-length hGCHII-GTP complex. These two files were then used to perform MD simulations. Simulations were carried out using pmemd.cuda module of AMBER14 (Pearlman et al., 1995). The system containing hGCHII-GTP complex was sequentially subjected to minimization, heating, equilibration, and production. In the first step of minimization, only explicit water molecules were allowed to adjust their orientations while, in second step all the system molecules were allowed to move to accommodate protein, GTP, ions, and water. Heating was performed using NVT ensemble throughout 100 ps whereby the temperature was stabilized at 300 K. Subsequently, the system was equilibrated for 10 ns to have a uniform density of 1 g/cm³ of water and a stable root mean square deviation (RMSD) of protein. The simulations then continued in an isothermal-isobaric ensemble (NPT, p = 1 atm, and T = 300 K) for 40 ns with the time step of 2 fs. The trajectory was recorded for every four ps. During simulation, pressure and temperature were controlled by using Berendsen barostat, and Langevin thermostat, respectively (Berendsen et al., 1984; Lontcharich et al., 1992). SHAKE algorithm was used to constrain hydrogen containing bonds (Van Gunsteren & Berendsen, 1977). The particle mesh Ewald method was applied to calculate long-range van der Waals interactions over the distance of 8 Å (Darden et al., 1993). The Pymol (The PyMOL Molecular Graphics System, Version 1.2r3pre. Schrödinger, LLC New York, NY) and VMD (Humphrey et al., 1996) were used for the visualization and analysis of the trajectories. The same simulation protocol was also used for mutant complexes.

**Binding free energy calculations of hGCHII complexes**

MM-PBSA and MM-GBSA methods were used to study binding affinity of GTP in full length and mutant complexes of hGCHII (Cheatham et al., 1998; Kuhn & Kollman, 2000). The binding free energy calculated by these methods was the addition of molecular mechanical energy (electrostatic energy, Van der Waals energy, and internal energy including bond, angle and torsional angle energy), polar solvation energy calculated either by Poisson-Boltzmann (PB) method with DELPHI program (Honig & Nicholls, 1995) or generalized Born (GB) method with Onufriev’s method (Onufriev et al., 2004) and nonpolar solvation energy calculated using solvent accessible surface area (SASA) computed by Linear Combinations of Pairwise Overlaps (LCPO) method (Weiser et al., 1999). The salt concentration was kept at 0.1 M. Binding free energy calculations were averaged over 500 frames taken at the interval of 80 ps over the production simulation of 40 ns. In addition, alanine scanning calculations on the binding free energy of GTP in hGCHII-GTP complex was performed for residues His-190, Leu-191, Leu-192, Tyr-180, and Lys-184. Each one of these residues was mutated to alanine, creating 5 different hGCHII-GTP complexes. These complexes along with hGCHII-GTP complex were subjected to MM-PBSA and MM-GBSA calculations using same protocol to analyze individual residue contribution toward the binding of GTP.

**Results**

**Cloning, purification and characterization of hGCHII and its deletion mutants**

The molecular model for last ~25 C-terminal residues of eGCHII, mGCHII, and hGCHII (Ren et al., 2005; Singh et al., 2013; Yadav & Karthikeyan, 2015) were not built in their respective crystal structures due to lack of electron density and therefore, considered as disordered region. To investigate the role of these structurally disordered C-terminal residues in the catalytic activity of hGCHII, various deletion mutants were constructed. In the absence of structural information for the C-terminal region of hGCHII, the deletion mutants were designed based on its predicted secondary structure. The primary sequence was used to predict the secondary structure of hGCHII (192 residues) by employing PSIPRED server (Bryson et al., 2005). The PSIPRED predicted the secondary structure elements for the C-terminal disordered region of hGCHII as a random coil (for residues 172–177), helix (for residues 178–187), and a random coil (for residues 188–192) (Figure 2). With the aim of not to disrupt
the secondary structure elements, three hGCHII deletion mutants namely hGCHII188 (residues 1–188), hGCHII177 (residues 1–177), and hGCHII171 (residues 1–171) were designed (Figure 3(A)). The hGCHII and its deletion mutants (hGCHII188, hGCHII177, and hGCHII171) were cloned, expressed, and purified as described in the experimental section. A typical SDS-PAGE profile of purified proteins is shown in Figure 3(B). The oligomeric status of hGCHII and its mutants were determined by analytical size exclusion chromatography and their elution profile is shown in Figure 3(C). The gel-filtration profile indicated that hGCHII and its deletion mutants existed as a dimer. To check if the C-terminal deletion mutants were folded well, we compared the CD spectra of mutant proteins with full-length hGCHII. The CD spectra showed similar pattern for both full-length and C-terminal deletion mutants suggesting that the mutant proteins were folded well (Figure 3(D)).

**Disordered C-terminal residues of GCHII were essential for catalytic activity**

To determine the functional aspects of hGCHII and its mutants, enzymatic assay was performed for these proteins. The catalytic activity of hGCHII was measured by monitoring the time course increase in absorbance at 310 nm for the formation of product, DARP. While hGCHII showed time based increase in absorbance, none of the mutant proteins (hGCHII188, hGCHII177, and hGCHII171) showed any significant increase in absorbance at 310 nm, indicating that the deletion mutants were unable to catalyze the formation of product, DARP (Figure 4(A)). Interestingly, loss of catalytic activity of hGCHII188 indicated that the last four C-terminal residues played a crucial role in functioning of the enzyme.

Further, to know whether these results would be valid for other bacterial counterparts, we extended this study for *E. coli* (eGCHII) and *S. typhimurium* (sGCHII) GCHII. eGCHII was specifically considered as biochemical and structural studies are already available in the literature and a mechanism has also been proposed for its catalysis. In addition to full-length, the C-terminal deletion mutants corresponding to hGCHII188, hGCHII177, hGCHII171 were generated for *E. coli* (eGCHII190, eGCHII179, eGCHII173) and *S. typhimurium* (sGCHII190, sGCHII179, sGCHII173) (Figure 4(B)). The cloning, expression and purification of eGCHII, sGCHII and their mutants were carried out as described in the experimental section. A typical SDS-PAGE profile for the purified proteins is shown in Figure 4(C). In the enzymatic assay, both eGCHII and sGCHII catalyzed the formation of DARP efficiently whereas their mutant proteins,
similar to hGCHII mutants, did not catalyze the formation of DARP (Figure 4(D)). Although eGCHII190 and sGCHII190 showed activity of about 5–8% relative to their full-length proteins, the other mutants (eGCHII179, eGCHII173, sGCHII179, and sGCHII173) were catalytically inactive.

**Homology models of hGCHII and its mutants**

The structural models of last C-terminal residues of eGCHII, mGCHII, and hGCHII were not resolved owing to the absence of electron density in that region and therefore, existed in disordered form. Interestingly, the deletion of merely last four amino acids of hGCHII rendered a catalytically inactive enzyme, thus, revealing the essentiality of C-terminal residues of hGCHII. However, the mechanistic details for such loss of enzymatic activity exhibited by the hGCHII mutants were not known. Thus, to understand the basis for loss of activity displayed by hGCHII mutants, we used molecular dynamics simulation studies. The hGCHII crystal structure (PDB ID: 4RL4) has been solved in the inactive state, where the catalytically essential active site cysteine residues (Cys-55, Cys-66, and Cys-68) were present in oxidized state forming disulfide bonds and the structure was also devoid of GTP, Zn²⁺, and Mg²⁺ (Yadav & Karthikeyan, 2015). Moreover, the superposition of hGCHII (PDB ID: 4RL4) and eGCHII crystal structures (PDB ID: 2BZ0) (Ren et al., 2005) revealed conformational deviations in the residues surrounding the active site. Therefore, to understand the role of C-terminal residues in the catalysis, we modeled a full-length structure of hGCHII (192 residues) by using the I-TASSER server (Yang et al., 2015) with GMPCPP bound crystal structure of eGCHII as template.

The I-TASSER server predicted five models for full-length hGCHII with a C-score value of 0.12, −0.42, −0.65, −0.37, and −1.30 (Figure 5(A)). The C-score value is a confidence score for estimating the quality of predicted models by the I-TASSER that lies in the range of −5 to 2. The higher value of the C-score meant the model with higher confidence. The overall structure of these five models, for the residues 1–172, was similar to hGCHII and eGCHII structures. However, the C-terminal region (residues 172–192) of these five predicted models varied in its orientation and the residue conformations (Figure 5(A)). We reasoned that these C-terminal residues either might play a role in the binding of GTP or in the catalysis or both as experimental findings have depicted their essentiality for the enzyme activity. Therefore, the top ranked model (model 1) in which the C-terminal residues were near to the active site was selected for further studies. The selected monomer showed RMSD of 1.33 Å (130 Cα atoms) and 0.63 Å (132 Cα atoms) with hGCHII (PDB ID: 4RL4) and eGCHII (PDB ID: 2BZ0), respectively (Figure 5(B)).
hGCHII monomer was further modeled with its substrate GTP and metal ions Mg$^{2+}$ and Zn$^{2+}$ as described in method section, hereafter referred as hGCHII-GTP complex. The quality of the final hGCHII model was checked by PROCHECK (Laskowski et al., 1993). The PROCHECK showed 90% residues of the hGCHII were in the most favorable region, while 10% were in the additionally favored region of Ramachandran plot. Various model quality related parameters are provided in supplementary material (Supplementary Figures S1–S6 and Table S1). Since, the hGCHII existed as a dimer in solution as...
well as in the crystal structure, the hGCHII-GTP-complex was converted to dimer by superposing its two monomers on eGCHII structure (Figure 5(C)). Dimer complexes of hGCHII188, hGCHII177, and hGCHII171 were then prepared by removing last 4-, 15- and 21-residues from the full-length hGCHII-GTP-complex structure.

The active site analysis of modeled hGCHII structure in complex with GTP revealed that the residues Arg-50, Glu-54, Glu-92, Arg-94, Gln-108, Thr-114, Arg-128, and Lys-154 were interacting with substrate GTP (Figure 5(D)). The triphosphate moiety of GTP was lined with positively charged residues Arg-50, Arg-128, and Lys-154. Arg-50 was interacting with \( \gamma \)-phosphate of GTP whereas Lys-154 was bridging the \( \gamma \)-phosphate and \( \beta \)-phosphate groups. In eGCHII structure, the interaction of Lys-154 and \( \gamma \)-phosphate of GMPCPP is mediated by hydrogen bond whereas in hGCHII-modeled complex with GTP, this interaction was observed as long-range interaction (3.9 Å). Closer to
-phosphate of GTP, an extensive network of charged (Arg-128, Asp-126, Arg-94) group interactions were observed. The residue Arg-128 was forming salt bridges to the \( \alpha \)- and \( \beta \)-phosphate groups of GTP and with the side chain of Asp-126. Arg-94 was interacting with the \( \alpha \)-phosphate group via hydrogen bond as well as with the oxygen of ribose (O-4) (3.9 Å) (Figure 5(D)). Furthermore, the ribose 3'-hydroxyl was hydrogen-bonded to Glu-54 main-chain carbonyl oxygen whereas the 2'-hydroxyl of ribose was interacting with carboxyl group of Glu-92 via van der Waals interactions (Figure 5(D)). The residues, Gln-108 and Thr-114 were interacting with O-6 of the guanine base. In earlier studies, it is speculated that such interactions may give rise to base selectivity of GCHII for GTP (Ren et al., 2005). The guanidinium group of Arg-128 was located between the \( \alpha \)- and \( \beta \)-phosphate groups of GTP, interacting via salt bridge and hydrogen bond, and in a position to act as the nucleophile (Ren et al., 2005). Mg\(^{2+}\) was interacting with the side chain of His-52 and \( \alpha \)- and \( \beta \)-phosphate group of GTP and thus, in a position to neutralize the negatively charged groups of the GTP. The other metal ion, Zn\(^{2+}\) was found to coordinate with three cysteine residues Cys-55, Cys-66 and Cys-68 and a water molecule similar to eGCHII structure (Ren et al., 2005).

### MD simulations showed the interactions mediated by C-terminal residues of hGCHII

All the modeled dimer complexes (hGCHII-GTP and its three mutants) were subjected to MD simulations. Trajectory and energetic analysis showed that hGCHII-GTP complex was stable. It had a uniformly fluctuating RMSD of \( \sim 4.0 \text{ Å} \) (Supplementary Figure S7). RMSDs of each mutant complex of hGCHII were also stable during respective MD trajectories (Supplementary Figure S8). Further, root mean square fluctuation (RMSF) analysis showed that C-terminal amino acid residues (171 onwards) were highly flexible as compared to rest of the residues (Supplementary Figure S9), correlating well with their disordered nature as observed in the crystal structures.

During MD simulations, in the monomer-1 subunit of hGCHII-GTP complex, GTP formed stable interactions with
the active site residues viz. Arg-50, Gly-93, Arg-94, Asn-118, Arg-128, and Lys-154. The structure of eGCHII in complex with substrate analogue also showed the same residues as a part of the binding site of GMPCPP (Ren et al., 2005). MD simulations additionally revealed two C-terminal residues, Tyr-180 and Lys-184, consistently interacting with the GTP (Figure 6(A)) and were found to have low values of RMSF for these residues (Supplementary Figure S9). Tyr-180 interacted via the formation of a hydrogen bond with the oxygen atom of γ-phosphate of GTP through its side-chain hydroxyl group (Figure 6(A,B)) and Lys-184 formed charge-charge interaction with γ-phosphate of GTP (Figure 6(A,C)). Interestingly, these residues were also conserved among different species (Supplementary Figure S10). Furthermore, the interactions of the last four residues viz. Gly-189, His-190, Leu-191, and Leu-192, which were shown to be indispensable for the catalytic activity of hGCHII were analyzed. In the initial structure of hGCHII-GTP, none of these residues were found to interact with GTP. However, as MD simulations progressed, His-190 moved from its position and initiated a hydrogen bond interaction with the α-phosphate oxygen of GTP through side-chain nitrogen (Figure 6(A)). Once formed, this interaction continued throughout the simulations (Figure 6(D)), as supported by low RMSF value for His-190. At the same time, a water molecule from the solvent approached and stabilized near α and γ-phosphate group, forming hydrogen bonds with their oxygen atoms (Figure 6(A)). Water molecules were observed within 3 Å of N° of His-190 for more than 88% of time of MD simulations while less number of water molecules (less than 77%) were found within 3 Å of N° of His-190 in both monomers of hGCHII. Three other residues (Gly-189, Leu-191, and Leu-192), among the last four residues of hGCHII, did not interact with GTP. The Gly-189 seemed to play a structural role, which allowed His-190 to move and interact with the α-phosphate oxygen atom, because of the absence of side chains. The Leu-191 made hydrophobic interactions with the side-chain carbon atoms of Ile-115, Asp-125, Asp-126, and Arg-94 (Figure 7(A)). Among these four interacting residues, Arg-94 and Asp-126 are conserved while Ile-115 is replaced with other hydrophobic residues such as valine, tyrosine, and phenylalanine in different bacteria (Supplementary Figure S10). Moreover, Leu-191 also made a hydrogen bond interaction with side chain nitrogen of Asn-151, through the backbone carbonyl oxygen atom (Figure 7(B)). Interestingly, the Asn-151 is conserved in GCHII among bacteria (Supplementary Figure S10). The last C-terminal residue of the hGCHII, Leu-192, did not show hydrophobic interaction with any of the residues of the enzyme, however, it formed a weak van der Waals interaction consistently with Lys-184 (Figure 7(C)) and also showed low RMSF value (Supplementary Figure S9). MD simulation of C-terminal deletion mutants complexes i.e. hGCHII171, hGCHII177, and hGCHII188 showed stable interactions of GTP with the non-C-terminal residues viz. Arg-50, Gly-93, Arg-94, Asn-118, Arg-128, and Lys-154, as observed in the full-length GCHII-GTP complex. Interactions between ligands and all the proteins at different times during MD simulations are presented in Supplementary Figures S11–S14. Interactions made by C-terminal residues with GTP were gradually reduced with an increase in number of deleted residues from hGCHII. The full-length hGCHII showed interactions of GTP with Tyr-180, Lys-184, and His-190, indicating the role of C-terminal residues in the stabilization of GTP. However, hGCHII178, hGCHII177, and hGCHII171 lacked these interactions with GTP. Thus, favorable energetic contribution of His-190 toward the binding of GTP was absent in all of the deletion mutant complexes. In order to affirm the importance of His-190, MD simulations with H190A mutated structure were also performed. The overall structure of H190A was found to be stable throughout the 50 ns of MD simulations. Although, Ala-190 was found to be unstable at its position due to lack of favorable interactions with GTP, it was transiently stabilized by the hydrophobic interactions with Lys-184 and Pro-152 side-chains. Consequently, it moved away from GTP toward the bulk solvent. To comprehend the energetic contribution of interactions of C-terminal residues with GTP, MM-PBSA and MM-GBSA binding free energies in full length and mutant complexes were computed (Table 2). The results revealed a recognizable difference in binding energies of the GTP in four complexes. GTP had −193.36 (MM-PBSA) and −208.37 kcal/mol (MM-GBSA) free energy of binding with full length complex. This energy decreased proportionately with the removal of number of C-terminal amino acid residues. In full length complex of hGCHII, His-190 formed a hydrogen bond with GTP (Figure 6(D)). However, this interaction could not be formed in hGCHII188-GTP complex because of His-190’s absence, as also reflected in ΔG binding (Table 2). Mutant hGCHII188-GTP complex lacking His-190 showed a difference in ΔG binding of GTP by −7 kcal/mol in MM-PBSA and −17 kcal/mol in MM-GBSA calculations, respectively as compared to full length hGCHII-GTP complex. Further, MM-PBSA and MM-GBSA calculations were performed on alanine mutants of His-190, Leu-191, Leu-192, Tyr-180, and Lys-184 in hGCHII-GTP complex to obtain energy contribution by these residues toward GTP binding (Table 3). Analysis of these contributions revealed a favorable energy contribution by His-190 toward its binding with GTP. Details of MM-PBSA and MM-GBSA binding free energies for all complexes and alanine mutants are given in supplementary Table S2 to Table S5. Thus, these results cumulatively confirmed that the His-190 interacts with GTP and may play an important role in binding of GCHII with GTP.

**Discussion**

*H. pylori*, responsible for the gastric cancer, is of high-priority among the list of antibiotic-resistant bacteria that pose the

| Mutation  | MM-PBSA (kcal/mol) | MM-GBSA (kcal/mol) |
|-----------|---------------------|--------------------|
| H190A     | −22.70              | −14.34             |
| L191A     | −1.24               | −1.37              |
| L192A     | −4.20               | −0.64              |
| Y180A     | −28.61              | −10.36             |
| K184A     | −74.76              | −34.84             |
greatest threat to human health as published by WHO (Dang & Graham, 2017). Owing to increase in the antimicrobial resistance, H. pylori infection is becoming difficult to cure and, therefore, there is a need for new targets/therapeutics to treat the infection. GCHII, one of the enzymes of the riboflavin biosynthesis pathway, is a potential drug target for the gastric pathogen H. pylori as this enzyme is essential for bacteria but absent in humans (Fassbinder et al., 2000). For designing a successful drug, a detailed knowledge about biochemical, biophysical, and structural properties of the drug target is required. Thus, to develop an inhibitor that can specifically target GCHII, we recently characterize this enzyme from H. pylori (hGCHII) (Yadav & Karthikeyan, 2015).

Biochemical and kinetic parameters of hGCHII are similar to other species such as E. coli (Foor & Brown, 1975; Kaiser et al., 2002; Ritz et al., 2001; Schramek et al., 2001), S. coelicolor (Spoonamore et al., 2006; Spoonamore & Bandarian, 2008), B. subtilis (Lehmann et al., 2009), and M. tuberculosis (Singh et al., 2013). However, unlike its bacterial counterparts, the crystal structure of hGCHII exhibits inactive state with its active site cysteine found in oxidized form and devoid of Mg2+ and Zn2+, which are indispensable for the catalytic activity (Yadav & Karthikeyan, 2015). Nevertheless, the superposition of three available GCHII crystal structures i.e. eGCHII (PDB ID: 2B20), mGCHII (PDB ID: 4I14), and hGCHII (PDB ID: 4RL4) reveal a same α/β fold with RMSD of 1.4–1.5 Å for 112 to 135 Cα atoms. Surprisingly, in all three crystal structures of GCHII, approximately last ~25-residues of C-terminal region remains disordered, although all of them are crystallized using full-length protein. Earlier, Ren et al. proposed a catalytic mechanism for GCHII of E. coli based on its biochemical and structural characterization (Ren et al., 2005). In the proposed mechanism, however, no function has been attributed to the last ~25 C-terminal residues and therefore, the role played by these residues in GCHII function is hitherto unknown. Thus, to investigate the role of C-terminal residues, we have initiated the enzymatic and structural studies focused on disordered C-terminal region of hGCHII. The hGCHII consists of 192 amino acid residues in its full-length, but, the crystal structure shows electron density only up to 172 amino acid residues (Yadav & Karthikeyan, 2015). Based on this observation, we define the residues from 173–192 as C-terminal disordered region of hGCHII. A secondary structure prediction using the full-length amino acid sequence of hGCHII reveal that the residues from 172–177 and 188–192 form random coil structures, while, the residues from 178 to 187 form a helix. Accordingly, we have constructed three C-terminal deletion mutants of hGCHII and named as hGCHII177 (residues from 1 to 171), hGCHII177 (residues from 1 to 177), and hGCHII188 (residues from 1 to 188). All the three mutants were cloned, expressed and purified similar to full-length hGCHII protein. These mutant proteins exist as dimer, as revealed by size-exclusion chromatography and show similar CD spectra to that of full-length indicating the deletion of C-terminal residues of hGCHII neither affect the dimerization nor its folding. Despite this, the three mutants (hGCHII177, hGCHII177, and hGCHII188) were unable to catalyze the formation of product DARP, indicating that these are enzymatically inactive. In fact, the hGCHII188 mutant, in which only last four amino acid residues were deleted, show only ~3% of catalytic activity as compared to full length hGCHII. These results strongly suggest that the disordered C-terminal residues of hGCHII are essential for its catalytic activity. Although, this result is surprising for hGCHII, it is not uncommon that a last few C-terminal residues play a role in the catalytic activity of enzyme (Washington et al., 2013). Nevertheless, to check whether these results can be extended to other GCHII enzymes, we have purified three C-terminal deletion mutant proteins of E. coli (eGCHII179, eGCHII176, eGCHII173) and S. typhimurium (sGCHII179, sGCHII177, sGCHII173) corresponding to hGCHII188, hGCHII177, and hGCHII177 proteins. Similar to hGCHII mutants, the mutant enzymes of E. coli and S. typhimurium are also unable to catalyze the formation of product DARP and thus, exist in catalytically inactive form.

Although, we have shown that the full-length GCHII is essential for its catalytic activity, the molecular mechanism for such strict requirement is unknown. In fact, in the proposed mechanism of eGCHII, the disordered C-terminal residues do not have any assigned function (Ren et al., 2005). Therefore, homology modeling and molecular dynamics (MD) simulation studies are employed to get mechanistic insights for the loss of activity shown by the mutants of hGCHII. A full-length dimeric model of hGCHII in complex with GTP, Zn2+, and Mg2+ (hGCHII-GTP) was built using I-TASSER server and subjected to MD simulations. MD simulation studies revealed a hydrogen bond interaction between α-phosphate oxygen of GTP and Nε of His-190 in the full-length of hGCHII-GTP complex. However, this interaction is not observed in all three C-terminal deletion mutants of hGCHII due to absence of His-190. To confirm the importance of this interaction, a hGCHII-GTP model, in which His-190 is replaced with Ala, was also subjected to MD simulations. These simulation studies clearly show that Ala-190 neither make any hydrogen bond with GTP nor contribute in free binding energy vis-a-vis His-190.

Earlier studies suggested that the Arg-128 may act as a nucleophile during the hydrolysis of pyrophosphate from GTP and may be involved in the formation of GCHII-GMP adduct during the ring opening step of the catalysis (Ren et al., 2005). However, the pyrophosphate hydrolysis, being a rate-limiting step, the arginine may not be an optimal residue for this role and it is an unusual residue for forming covalent intermediate involving guanylation or adenylation (Ren et al., 2005). In fact, histidine could possibly act as a nucleophile and may form a covalent adduct as reported in case of Human histidine triad nucleotide binding protein 1 (hHint1) (Zhou et al., 2013). Thus, in the present study, we propose that His-190 may get involved in formation of hGCHII-GMP adduct instead of Arg128-GMP adduct, because of its closeness to the α-phosphonate as compared to Arg-128 and histidine is also known to form the covalent linkage with 3′-phosphate-DNA (He et al., 2007; Raymond et al., 2004). In addition, His-190 may act as a proton acceptor, leading to the activation of the water molecule which supposedly carries out the hydrolysis of the enzyme-DARP covalent adduct, setting free enzyme and product, DARP.
presence of a water molecule in the vicinity of His-190 during the simulations also supports this hypothesis. All these results strongly suggest that His-190, a conserved residue, plays a crucial role in the catalytic activity of hGCHII. Taken together these results demonstrate that the unstructured C-terminal residues of GCHII are important for its enzymatic activity and therefore, full-length GCHII must be considered during rational drug designing.

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Author contribution

SK and SY conceived the idea. SY and SS performed, interpreted, and analyzed the experimental studies. RS performed and analyzed the MD simulation studies. BS supervised the simulation studies. All authors contributed in writing and proof reading of the manuscript.

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References

Berendsen, H. J. C., Postma, J. P. M., van Gunsteren, W. F., DiNola, A., & Haak, J. R. (1984). Molecular dynamics with coupling to an external bath. Journal of Chemical Physics, 81(8), 3684–3690. https://doi.org/10.1063/1.448118

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248–254. https://doi.org/10.1016/0003-9861(76)90527-3

Bryson, K., McGun, L. J., Marsden, R. L., Ward, J. S., Soda, J. J., & Jones, D. T. (2005). Pspred protein prediction server. Protein structure prediction servers at University College London. http://bioinf.cs.ucl.ac.uk/psipred

Cheatham 3rd, T. E., Srinivasan, J., Case, D. A., & Kollman, P. A. (1998). Molecular dynamics and continuum solvent studies of the stability of polyG-polyC and polyA-polyT DNA duplexes in solution. Journal of Biomolecular Structure and Dynamics, 16, 265–280. https://doi.org/10.1080/07391102.1998.10508245

Dang, B. N., & Graham, D. Y. (2017). Helicobacter pylori infection and antibiotic resistance: A WHO high priority? Nature Reviews Gastroenterology & Hepatology, 14, 383–384. https://doi.org/10.1038/nrgastro.2017.57

Darden, T., York, D., & Pedersen, L. (1993). Particle mesh Ewald: An N-log(N) method for Ewald sums in large systems. Journal of Chemical Physics, 98(12), 10089–10092. https://doi.org/10.1063/1.464397

Fassbinder, F., Kist, M., & Bereswill, S. (2000). Structural and functional analysis of the riboflavin synthetase genes encoding GTP cyclohydrolase II (ribA), DHBP synthase (ribBA), riboflavin synthase (ribC), and riboflavin deaminase/reductase (ribD) from Helicobacter pylori strain P1. FEMS Microbiology Letters, 191, 191–197. https://doi.org/10.1111/j.1574-6968.2000.tb09339.x

Foor, F., & Brown, G. M. (1975). Purification and properties of guanosine triphosphate cyclohydrolase II from Escherichia coli. Journal of Biological Chemistry, 250, 3545–3551. https://doi.org/10.1016/S0021-9258(19)41549-4

Foor, F., & Brown, G. M. (1980). GTP cyclohydrolase II from Escherichia coli. Methods in Enzymology, 66, 303–307.

He, X., van Waardenburg, R. C. A. M., Babaoglu, K., Price, A. C., Nittis, K. C., Nittis, J. L., Bjornsti, M. A., & White, S. W. (2007). Mutation of a conserved active site residue converts tyrosyl-DNA phosphodiesterase I into a DNA topoisomerase I-dependent poison. Journal of Molecular Biology, 372, 1070–1081. https://doi.org/10.1016/j.jmb.2007.07.055

Honig, B., & Nicholls, A. (1995). Classical electrostatics in biology and chemistry. Science, 268(5214), 1144–1149. https://doi.org/10.1126/science.7761829

Humphrey, W., Dalke, A., & Schulten, K. (1996). VMD: Visual molecular dynamics. Journal of Molecular Graphics, 14, 33–38. https://doi.org/10.1016/0263-7855(96)00018-5

Jorgensen, W. L., Chandrasekhar, J., Madura, J. D., Impey, R. W., & Klein, M. L. (1989). Comparison of simple potential functions for simulating liquid water. Journal of Chemical Physics, 79(2), 926–935. https://doi.org/10.1063/1.445869

Kaiser, J., Schramek, N., Eberhardt, S., Puttmann, S., Schuster, M., & Bacher, A. (2002). Biosynthesis of vitamin B2: An essential zinc ion at the catalytic site of GTP cyclohydrolase II. European Journal of Biochemistry, 269, 5264–5270. https://doi.org/10.1046/j.1432-1033.2002.03239.x

Kuhn, B., & Kollman, P. A. (2000). Binding of a diverse set of ligands to avidin and streptavidin: An accurate quantitative prediction of their relative affinities by a combination of molecular mechanics and continuum solvent models. Journal of Medicinal Chemistry, 43, 3786–3791. https://doi.org/10.1021/jm000241n

Laskowski, R. A., MacArthur, M. W., Moss, D. S., & Thornton, J. M. (1993). PROCHECK: A program to check the stereochemical quality of protein structures. Journal of Applied Crystallography, 26(2), 283–291. https://doi.org/10.1107/S0021889892009944

Lehmann, M., Degen, S., Hohmann, H. P., Wyss, M., Bacher, A., & Schramek, N. (2009). Biosynthesis of riboflavin. Screening for an improved GTP cyclohydrolase II mutant. FEBS Journal, 276, 4119–4129. https://doi.org/10.1111/j.1742-4658.2009.07118.x

Li, P., Roberts, B. P., Chakravorty, D. K., & Merz, K. M. (2013). Rational design of particle mesh ewald compatible Lennard-Jones parameters for -2 metal cations in explicit solvent. Journal of Chemical Theory and Computation, 9, 273–2748. https://doi.org/10.1021/ct400146w

Loncharich, R. J., Brooks, B. R., & Pastor, R. W. (1992). Langevin dynamics and Computation. 1111/j.1742-4658.2009.00946.x

Maier, A. J., Martinez, C., Kasavajhala, K., Wickstrom, L. Hauser, K. E., & Simmerling, C. (2015). ff14SB: Improving the accuracy of protein side chain and backbone parameters from ff99SB. Journal of Applied Crystallography, 48, 2523–2535. https://doi.org/10.1107/S0021-9727-1504830

Meagher, K. L., Redman, L. T., & Carlson, H. A. (2003). Development of polyphosphate parameters for use with the AMBER force field. Journal of Chemical Theory and Computation, 11, 3696–3713. https://doi.org/10.1021/acs.jctc.0b00255

Onufriev, A., Bashford, D., & Case, D. A. (2004). Exploring protein native states and large-scale conformational changes with a modified
generalized Born model. *Proteins*, 55, 383–394. https://doi.org/10.1002/prot.20033

Pearlman, D. A., Case, D. A., Caldwell, J. W., Ross, W. S., Cheatham, T. E., DeBoit, S., Ferguson, D., Seibel, G., & Kollman, P. (1995). AMBER, a package of computer programs for applying molecular mechanics, normal mode analysis, molecular dynamics and free energy calculations to simulate the structural and energetic properties of molecules. *Computer Physics Communications*, 91(1–3), 1–41. https://doi.org/10.1016/0010-4655(95)00041-D

Raymond, A. C., Rideout, M. C., Staker, B., Hjerrild, K., & Burgin, A. B. Jr. (2004). Analysis of human tyrosyl-DNA phosphodiesterase I catalytic residues. *Journal of Molecular Biology*, 338, 895–906. https://doi.org/10.1016/j.jmb.2004.03.013

Ren, J., Kotaka, M., Lockyer, M., Lamb, H. K., Hawkins, A. R., & Stammers, D. K. (2005). GTP cyclohydrolase II structure and mechanism. *Journal of Biological Chemistry*, 280, 36912–36919. https://doi.org/10.1074/jbc.M507725200

Ritz, H., Schramek, N., Bracher, A., Herz, S., Eisenreich, W., Richter, G., & Bacher, A. (2001). Biosynthesis of riboflavin: Studies on the mechanism of GTP cyclohydrolase II. *Journal of Biological Chemistry*, 276, 22273–22277. https://doi.org/10.1074/jbc.M100752200

Schramek, N., Bracher, A., & Bacher, A. (2001). Biosynthesis of riboflavin. Single turnover kinetic analysis of GTP cyclohydrolase II. *Journal of Biological Chemistry*, 276, 44157–44162. https://doi.org/10.1074/jbc.M107036200

Singh, M., Kumar, P., Yadav, S., Gautam, R., Sharma, N., & Karthikeyan, S. (2013). The crystal structure reveals the molecular mechanism of bifunctional 3, 4-dihydroxy-2-butane 4-phosphate synthase/GTP cyclohydrolase II (Rv1415) from *Mycobacterium tuberculosis*. *Acta Crystallographica Section D: Biological Crystallography*, 69, 1633–1644. https://doi.org/10.1107/S0907444913011402

Spoonamore, J. E., & Bandarian, V. (2008). Understanding functional divergence in proteins by studying intragenomic homologues. *Biochemistry*, 47, 2592–2600. https://doi.org/10.1021/bi702263z

Washington, E. J., Banfield, M. J., & Dangl, J. L. (2013). What a difference a Dalton makes: Bacterial virulence factors modulate eukaryotic host cell signaling systems via deamidation. *Microbiology and Molecular Biology Reviews*, 77, 527–539. https://doi.org/10.1128/MMBR.00013-13

Willyard, C. (2017). The drug-resistant bacteria that pose the greatest health threats. *Nature*, 543, 15. https://doi.org/10.1038/nature.2017.21550

Yang, J., Yan, R., Roy, A., Xu, D., Poisson, J., & Zhang, Y. (2015). The I-TASSER Suite: Protein structure and function prediction. *Nature Methods*, 12, 7–8. https://doi.org/10.1038/nmeth.3213