Summary

Helicobacter pylori (*H. pylori*), a gram-negative bacterium, colonizes the human gastric mucosa, primarily responsible for stomach inflammation (chronic gastritis), peptic ulcer disease. It is one of the major risk factors for MALT-lymphoma and gastric cancer. Eradication of *H. pylori* significantly decreases the risk of developing cancer in infected individuals without pre-malignant lesions emphasizing the belief that this organism influences early stages in gastric carcinogenesis. Prevalence of antibiotic resistance is the main factor that contributes to the failure of current therapeutic regimens. Therefore, there is a urgent need to identify new drug targets. The enzymes involved in riboflavin biosynthesis pathway are shown to be essential for the survival of *H. pylori*. However, no structural information is available for these enzymes in *H. pylori*. In the present study, we have focused on the GTP cyclohydrolase II from *H. pylori* (hGCHII), one of the enzyme involved in riboflavin biosynthesis pathway, to understand its molecular mechanism and to explore it as a potential antibacterial drug target.

GTP cyclohydrolase II (GCHII) catalyzes the conversion of GTP to formation of 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinone-5′-phosphate (DARP), pyrophosphate (PP_i), and formate ($HCOO^-$). The proposed reaction mechanism for GCHII comprises release of pyrophosphate followed by imidazole ring opening, elimination of formate, and finally formation of main product DARP. In addition, GMP is also shown to be an alternate product of DARP at the molar ratio of 10:1. Here, we have cloned, expressed and purified the hGCHII protein by using Ni-NTA followed by size exclusion chromatography techniques. The size exclusion chromatography studies reveal that the hGCHII exists as dimer in solution. The far-UV CD study shows that the hGCHII is well folded and contains mixed helical and sheet content. Further, the thermal stability studies indicate that the protein is structurally stable up to 50°C and its melting temperature (T_m) is around 60°C. The calculated K_m, V_{max} and k_{cat} values are 23 ± 3 μM, 37 ± 2 nmol/min/mg and 0.89 min^{-1}, respectively. The catalytic activity studies on hGCHII show that the enzyme is specific for its substrate GTP and shows activity in broad range of pH from 7.0 to 10.0 with optimum activity at pH 8.0 and at 50°C. The enzyme activity of hGCHII with different metal ions shows that it requires Mg^{2+} for optimum activity while other metal ions such as Mn^{2+} and Ca^{2+} shows relatively less activity. We have solved the crystal structure of hGCHII in apo form at 2.20 Å resolution. Analysis of hGCHII crystal structure reveals that it belongs to α/β fold and forms mixed parallel and anti-parallel β-sheet. Surprisingly, the hGCHII structure is

devoid of any intrinsically bound Zn^{2+} and the catalytic cysteines are involved in disulfide bond formation, thus exits in oxidized form. However, the PAR assay in the presence of hydrogen peroxide (H_2O_2), shows that Zn^{2+} is present intrinsically in hGCHII, indicating that cysteines exist in reduced from. Mutational studies on all four cysteine (Cys-55, Cys-66, Cys-68, and Cys-172) residues, involved in disulfide bond formation, show that only Cys-55, Cys-66 and Cys-68 residues are required for the catalytic activity of the enzyme whereas fourth cysteine (Cys-172) can be dispensable for the catalytic activity of the hGCHII. Treatment of hGCHII with MMTS (a thiol modifying reagent) shows that cysteines at the catalytic site are oxidized, however, in the presence of reducing agent such as DTT, hGCHII is and able to go back to reduced form by taking Zn^{2+} from environment, confirmed by mass spectrometry studies on MMTS. Thus, these results strongly suggest that hGCHII can switch from reduced (active) to oxidized (inactive) form depending upon its redox environment and vice-versa with respect to its GCHII activity.

In all three GCHII crystal structures available from *H. pylori* (PDB ID: 4RL4), *E. coli* (PDB ID: 2BZ0), and *M. tuberculosis* (PDB ID: 4I14), the last C-terminal 20-25 residues remain disordered. To understand their role in the catalytic activity of hGCHII, if any, we have designed three C-terminal deletion mutants (hGCHII-171, hGCHII-177 and hGCHII-188) of hGCHII based on the secondary structure prediction. All the deletion mutant proteins are cloned, expressed and purified to homogeneity. The enzyme activity assay shows that all deletion mutant proteins (hGCHII-171, hGCHII-177 and hGCHII-188) are catalytically inactive and thus not able to catalyze the formation of DARP. However, the CD studies show that all proteins are well folded and contain secondary structure similar to wild type hGCHII, indicating that deletion of C-terminal residues has not affected the folding and stability of the hGCHII protein. The crystal structure of hGCHII-188 is solved upto 2.6 Å resolution in truncated form (residues 1-173) and the overall crystal structure of the hGCHII-188 remains similar to the wild type hGCHII in oxidized state. From these studies, it is concluded that residues (173-192) which remain disordered in hGCHII as well as in hGCHII-188 structure, are contributing to the catalytic activity of the enzyme.

In the absence of hGCHII structure in reduced form, we have modeled the hGCHII in reduced form and in complex with substrate GTP and two metals (Mg^{2+} and Zn^{2+}), which are shown to be indispensable for the catalytic activity. The active site residues are predicted with the help of hGCHII modeled structure. The mutational studies for the predicted active site residues are carried out to get detail insights of role of these residues in the catalytic

activity of enzyme. The hGCHII mutants for all predicted active site residues are constructed by using site directed mutagenesis approach and characterized. Most of the hGCHII mutants are good in expression and purified to homogeneity as of wild type with few exceptions like hGCHII-R50A, hGCHII-H52A, hGCHII-E54A and hGCHII-R94A. Although, the hGCHII-R50A, hGCHII-E54A and hGCHII-R94A proteins are good in expression, solubility of protein remains less as compared to wild type. The mutant protein hGCHII-H52A is not expressed at all and can be considered as crucial for hGCHII structurally as well as catalytically. The CD profile of most of the hGCHII mutants shows that they are well folded as similar to wild type hGCHII whereas mutants like hGCHII-R50A, hGCHII-E54A, hGCHII-Y105A and hGCHII-Y105F showed significant alteration in the secondary structure content as compared to wild type. Structural analysis of active site residues show that besides having role in catalysis, these residues are also interacting with other non-catalytic residues via hydrogen bonds and salt bridges and thus stabilizing the structure. However, none of the hGCHII mutants is found to be catalytically active, except hGCHII-D126E and hGCHII-Q108A which remains partially active. Thus, from these results, it can be concluded that all the active residues of the GCHII, which remain conserved across bacteria, are important for the catalytic activity of the hGCHII as evident by experimental validation and therefore employ the similar mechanism of catalysis as reported earlier for eGCHII.