GTP Cyclohydrolase II Structure and Mechanism*

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Jingshan Ren 1, Masayo Kotaka 1, Michael Lockyer 5, Heather K. Lamb 6, Alastair R. Hawkins 6, and David K. Stammers 2,3

From the 1Division of Structural Biology, The Wellcome Trust Centre for Human Genetics, University of Oxford, Roosevelt Drive, Oxford, OX3 7BN, 2Biological Section, Arrow Therapeutics Ltd., Trinity Street, Borough, London, SE1 1DA, and 3Institute of Cell and Molecular Sciences, Catherine Cookson Building, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne, NE2 4HH, United Kingdom

GTP cyclohydrolase II converts GTP to 2,5-diamino-6-β-ribosyl-4(3H)-pyrimidinone 5′-phosphate, formate and pyrophosphate, the first step in riboflavin biosynthesis. The essential role of riboflavin in metabolism and the absence of GTP cyclohydrolase II in higher eukaryotes makes it a potential novel selective antimicrobial drug target. GTP cyclohydrolase II catalyzes a distinctive overall reaction from GTP cyclohydrolase I; the latter converts GTP to dihydroneopterin triphosphate, utilized in folate and tetrahydrobipterin biosynthesis. The structure of GTP cyclohydrolase II determined at 1.54-Å resolution reveals both a different protein fold to GTP cyclohydrolase I and distinct molecular recognition determinants for GTP; although in both enzymes there is a bound catalytic zinc. The GTP cyclohydrolase II-GMPCPP complex structure shows Arg128 interacting with the α-phosphate, and thus in the case of GTP, Arg128 is positioned to act as the nucleophile for pyrophosphate release and formation of the proposed covalent guanylyl-GTP cyclohydrolase II intermediate. Tyr105 is identified as playing a key role in GTP ring opening; it is hydrogen-bonded to the zinc-activated water molecule, the latter being positioned for nucleophilic attack on the guanine C-8 atom. Although GTP cyclohydrolase I and GTP cyclohydrolase II both use a zinc ion for the GTP ring opening and formate release, different residues are utilized in each case to catalyze this reaction step.

The GCHII reaction involves a guanine ring-opening step and formate release, in common with the similarly named GTP cyclohydrolase I (EC 3.5.4.16) (GCHI), but other components of the two reactions catalyzed are rather different. GCHI does not hydrolyze pyrophosphate from GTP (unlike GCHII) but catalyzes a net expansion of the guanine base via a series of steps, starting with an opening of the imidazole ring and elimination of formate. An Amadori rearrangement involving the ribose is the next step, and finally ring closure yields a pteridine derivative, dihydroneopterin triphosphate (3). Dihydroneopterin triphosphate is utilized in microorganisms and plants as a folate precursor and in animals for the synthesis of tetrahydrobipterin, an essential cofactor for many enzymes (4), including nitric-oxide synthases. Crystal structure data show that a zinc ion is present in bacterial and human GCHI, which catalyzes the guanine ring opening via activation of a water molecule (5). Evidence from biochemical and mutagenesis studies indicates that GCHII also contains a zinc ion, which appears essential for guanine ring opening but not for pyrophosphate release (6). GCHII has no significant amino acid sequence identity with GCHI and forms a dimer compared with a homodimer in the case of GCHI (7).

The early work that first identified GCHII showed that, in addition to the formation of DARP, formate and pyrophosphate were also produced (8). Recent kinetic studies have provided further insights into aspects of the GCHII reaction mechanism (9, 10). The apparent production of further heterocyclic species from the GCHII reaction has been shown to be, in one case, the result of an in vitro artifact involving an anomerization step to give the α-isomer of DARP (9). The GCHII reaction does, however, yield an authentic alternative product to DARP, GMP, at a molar ratio of 10:1. 18O from solvent is incorporated into the phosphate group of DARP but not into the initially released pyrophosphate (9). Such kinetic data, along with the lack of substrate activity for GMP (8), have been interpreted as being consistent with the formation of a covalent guanylated GCHII intermediate. It is proposed that hydrolysis of this intermediate can occur either before or after ring opening to yield GMP or DARP, respectively. Single turnover kinetic analyses have revealed that the rate-determining step is at the beginning of the reaction pathway, presumed to be the formation of the covalent phosphoguanosine intermediate (10). A ring-opened guanine formamidine derivative of GTP, (2-amino-5-formylamino-6-ribosylamino-4(3H))-pyrimidinone triphosphate), has been shown to act as a substrate for GCHII, although it does not fulfill the criteria for a kinetically competent intermediate (11). Both GCHI and GCHII have much lower turnover numbers (between 1 and 5 min–1) than normally found for most enzymes (9).

Many years of overuse and misuse of antibiotics have turned the problem of antibiotic drug resistance into a crisis (12). One strategy for overcoming such issues is the development of a range of new antibacterial drugs against novel target sites, where preexisting resistance is not present. GCHII represents such a potential drug target, as ribofla-
vins are essential cellular coenzymes and many microorganisms do not possess a carrier system for the uptake of these molecules. GCHII is not present in higher eukaryotes, and thus low host toxicity for inhibitors of the enzyme might be anticipated. Knowledge of the three-dimensional structure along with detailed stereochromy of the enzyme active site will be of benefit in the structure-based design of inhibitors of GCHII. Although structural data for GCHII have been published (5, 13), which have led to proposals for the reaction mechanism (3, 11), there have been no crystal structures available for GCHII. Thus the question of whether there are any parallels between GCHII and GCHI in terms of three-dimensional structure and amino acid side-chains catalyzing the ring-opening mechanism has been unanswered to date. We therefore have determined a high resolution structure of *E. coli* GCHII as well as a structure with a bound GTP substrate analogue, GMPCPP. The data provide details of the pyrophosphate elimination and ring-opening reaction mechanisms in GCHII and allow comparisons with GCHI.

**MATERIALS AND METHODS**

Cloning, Expression, and Purification of GCHII—The gene for GCHII was amplified by PCR from *E. coli* genomic DNA using primers incorporating NdeI/BamHI restriction sites, and the product was ligated into the expression plasmid pRSETB (Invitrogen). The resulting plasmid, pRF87, coding for untagged GCHII, was transformed into the expression strain BL21(DE3)pLysS. Cells were grown as a 500-ml overnight culture in LB with ampicillin/chloramphenicol selection and were induced with isopropyl-1-thio-D-galactopyranoside for 5 h. 25 g of pelleted cells were sonicated in 450 ml of 50 mM potassium phosphate, pH 7.2, 2 mM DTT, 2 mM MgSO₄, 1 mM benzamidine. The lysate was clarified by centrifugation at 37 °C in a prewarmed rotor and centrifuge and washed three times with a total volume of 400 ml of 1× M9 minimal medium lacking a carbon source at 37 °C. The washed cells were then used to inoculate 10 × 500 ml of Athena minimal medium in 2-liter flasks, prewarmed to 37 °C, containing 40 µg ml⁻¹ selenomethionine. The cultures were induced with isopropyl-1-thio-β-D-galactopyranoside, and the incubation continued for a further 5 h, at which point the cells were harvested by centrifugation at 4 °C. Purification of selenomethionine-labeled GCHII used the same procedure as for the methionine protein except that the buffers contained 5 mM DTT.

Crystalization—GCHII at 24 mg/ml was subjected to a robotic screen of 672 crystallization conditions using the system developed in the Oxford Protein Production Facility (15). Protein droplets of 100 nl were mixed with 100 nl of reservoir solution in 96-well Greiner plates using a Cartesian Technologies pipetting station. Trays were incubated in an automated storage vault (The Automation Partnership, Royston, UK) at 22 °C and imaged initially once per day, using a Veeco visualization system. Hexagonal bipyramids crystals of unliganded GCHII were observed growing with ammonium sulfate as precipitant. Optimized crystallization conditions for both native and selenomethionine-labeled protein were 1.8 M ammonium sulfate, 0.1 M HEPES, pH 7.0, 15% glycerol, and 2% polyethylene glycol 4000, which reproducibly yielded large crystals within 24 h. Screening conditions for co-crystallization of GCHII with the GTP analogue, GMPCPP, gave crystals from 0.05 M ammonium acetate, 0.1 M bis-tris, pH 5.5, and 10% (w/v) polyethylene glycol 10,000.

**Data Collection, Selenium SAD Phasing, and Model Refinement**—Data were collected at beamline BM14, ESRF, for selenomethionine-labeled GCHII crystals frozen and maintained at 100 K. A fluorescence scan was used to set the optimal peak wavelength for the selenium edge. Data were indexed and integrated with DENZO (16). The SHELX program suite (17) was used to evaluate the quality of the anomalous signal during the course of the data collection. Following data preparation with SHELXC, SHELXD was used to identify three selenium sites and a metal site. Electron density maps calculated with SHELXE without further density modification using selenium peak SAD data were of high quality; thus data collection was terminated for the inflection wavelength. Automated model building was carried out with ARP/wARP (18).

Diffraction data for the tetragonal crystal form of GCHII with the bound GTP analogue were collected using Cu Kα radiation from an in-house Rigaku MicroMax 007 generator equipped with a MAR345 image plate. The structure was solved by molecular replacement with refined coordinates from the GCHII hexagonal crystal form using crystallography NMR software (CNS) (19). The GCHII structures were
refined in crystallography NMR software using simulated annealing, positional, and B-factor refinement to give the final statistics shown in TABLE ONE. O was used for model rebuilding (20).

**RESULTS**

**Structure Determination, Overall Fold of GCHII, and Relationship to Other Proteins**—Unusually, the selenomethionine-labeled crystals of GCHII diffracted to significantly higher resolution (1.54 Å) than did crystals of the methionine protein (2.0 Å), and thus data from the former were used for structural refinement. High quality phase angles were obtained using single wavelength anomalous scattering data collected at the selenium edge, as demonstrated by the electron density map from SHELXE (Fig. 1a). Interpretation of the electron density map for GCHII thus proved facile; much of the amino acid sequence could be read directly from the map. Indeed, automated model building with Arp/warp resulted in the fitting of 167 of 173 ordered residues, there being no density visible for the C-terminal region. Data collection, phasing, and refinement statistics are shown in TABLE ONE. GCHII has an α-β fold with a central core of mainly antiparallel β-sheet interconnected by loops and helices (Fig. 1, c–e). Residues 91–129 form an extended region positioned away from the main body of the protein and contain two helices, α-2 and α-3. Contacts for a possible GCHII dimer observed across a crystallographic 2-fold in the hexagonal form were also seen within the dimer in the asymmetric unit for the tetragonal crystal form (Fig. 1e).

DALLI searches showed relatively low levels of relatedness of GCHII to other known structures. The closest match was for hydrosolylase (root mean square deviation for 109 equivalent α-carbons 3.6 Å, Z score = 5.4, for the A chain of Protein Data Bank entry 1GXS (23)). GCHII did not appear on the list of significant matches found by DALLI using GCHII coordinates as the search molecule, thus indicating that the two enzymes are of unrelated protein folds.

**Active Site of GCHII**—The initial electron density maps clearly showed that a metal ion was present in GCHII situated in a buried position between β-4 and α-1 (Fig. 1a), coordinated by three cysteine residues (Cys^54, Cys^65, and Cys^67) and a water molecule. The coordination geometry and bond lengths from sulfur to the metal were consistent with it being zinc (24). An x-ray fluorescence scan at the ESRF BM14 for a GCHII crystal also showed a peak at 9666.91 eV, a characteristic energy for zinc (data not shown).

It did not prove possible to bind substrate or inhibitors to the hexagonal crystal form of GCHII either by use of co-crystallization or soaking pregrown crystals. Two sulfate ions as well as a sulfoxone-containing fragment of HEPEs were identified in GCHII at what was later identified as the substrate site. The ammonium sulfate used as precipitant, together with the buffer component, was present at a high concentration in the crystallization media and thus may be competing for substrate/inhibitor binding. Rescreening of crystallization conditions for the complex of GCHII with GMPCPP resulted in a new tetragonal crystal form grown with polyethylene glycol as precipitant (rather than from ammonium sulfate), as used for the unliganded enzyme crystallization. Structure determination of the tetragonal form of GCHII revealed that the nucleotide was indeed bound in this case (Fig. 1b).

GMPCPP binds to GCHII via a number of mainly hydrophilic interactions (Fig. 2a). The triphosphate-binding part of the substrate pocket is lined with positively charged residues, Arg^49, Arg^129, and Lys^154. There is a magnesium ion that, as well as interacting with the side chain of His^73, also bridges the α- and β-phosphate groups of GMPCPP. The equivalent interaction in GTP would promote nucleophilic attack on the β-phosphorous atom resulting from charge neutralization, consistent with the requirement of magnesium for enzyme activity. Both Gln^70 and Arg^91 interact with the γ-phosphate of GMPCPP, whereas Lys^154 bridges the γ-phosphate and β-phosphate groups. Closer to the α-phosphate there is an extensive network of charged group interactions involving Arg^128, Asp^126, Arg^94, and the substrate analogue phosphonates. Arg^128 forms salt bridges to the α- and β-phosphate groups of GMPCPP from the opposite direction to the position of Gln^70. Arg^94, in turn, also forms a salt bridge with the side chain of Asp^126. Arg^94 interacts with the α-phosphate group but additionally stacks above the guanine ring of GMPCPP. On the opposite side of the guanine ring are Lys^101 and Tyr^105; the latter forms a hydrogen bond with the water molecule that is activated by the zinc ion.

Protein interactions with the GMPCPP ribose and guanine groups include both main-chain and side-chain contacts. The ribose 3'-hydroxyl is hydrogen-bonded to the Glu^188 main-chain CO, whereas the 2'-hydroxyl forms van der Waals interactions with the Gly^44 NH, the carboxyl group of Glu^120, and the amino group of Lys^101. Such rather long contact distances to the 2'-hydroxyl mean that they do not contribute much to the binding energy for the substrate, consistent with the observed activity of GCHII for dGTP (6). Arg^94 hydrogen-bonds to the ribose ring oxygen (O-4). There is a hydrogen bond from Thr^115 to the O-6 of the guanine base and also a pair of contacts between the side chain of Glu^108 and the guanine O-6 and NH2 groups. Such interactions may give rise to the base selectivity of the enzyme for GTP.

There are some small shifts in the positions of certain side chains adjacent to GMPCPP, observed on binding the nucleotide e.g. Arg^94, Lys^101, and Tyr^105 with maximum displacements of 2.3, 1.5, and 1.0 Å, respectively. However, no major conformational changes in GCHII on the binding of GMPCPP are observed, despite the different crystal forms used for comparison of unliganded and inhibitor-bound species. Thus, the extended region (residues 95–129) does not move to close the active site region, and the C-terminal 23 residues remain disordered in the liganded GCHII complex.

**Catalytic Mechanism of GCHII**—The delineation of the detailed stereochemistry of the GCHII active site, together with interactions of the substrate analogue GMPCPP, allows proposals for the mechanisms of different steps in the enzyme reaction. The first step in the GCHII mechanism following the binding of GTP is nucleophilic attack on the α-phosphorus of GTP to release pyrophosphate. The guanidinium group of Arg^126 is located between the α- and β-phosphate groups of GMPCPP, interacting via salt bridge and hydrogen bond formation. Arg^126 is thus in a position to act as the nucleophile and, if a covalent intermediate is formed as predicted previously (10), to produce the guanylated enzyme (Fig. 2a). A magnesium ion is positioned between the α- and β-phosphate groups of GMPCPP, which acts to neutralize the negatively charged groups of the nucleotide. In the case of GTP, a partial positive charge will be induced on the α-phosphorus atom and thereby promote nucleophilic attack by Arg^126. The carboxylate of Asp^126 forms a salt bridge with Arg^246, increasing the nucleophilicity of the guanidinum group. Asp^126 may also act as the proton acceptor for the puta-
tive guanylation reaction, although pyrophosphate itself may have this role. Lysine is commonly utilized to form covalent nucleotide complexes in many enzymes. The nearest lysine to the H9251 -phosphorus atom of GMPCPP in GCHII, Lys154, is more than 6 Å away, and thus not close enough for this role.

The second step in the GCHII reaction is the ring opening of the guanine group, a proposed mechanism for which is shown in Fig. 3. In the unliganded enzyme, as well as the complex with GMPCPP, the fourth zinc coordination position contains a water molecule that is polarized by the metal, increasing its acidity and thus producing a nucleophile (Fig. 3, a and c). This water, hydrogen-bonded to the hydroxyl of Tyr105, is located close to the guanine imidazole moiety and positioned for nucleophilic attack on the C-8 atom. We propose that Tyr105 acts as the proton donor to the N-7 atom of GTP; there is no nearby histidine residue, for example, to have this role. There is a second water molecule, linked to the Tyr105 hydroxyl, which is involved in a network of further hydrogen bonds linking to Glu92 via Thr56 and Lys101 (Fig. 2a). Rearrangement of the substrate intermediate after water addition allows ring opening, leading to the putative formamide derivative (Fig. 3, b and c). A second round of water addition from the zinc ion can

FIGURE 1. Structure of GCHII. a, electron density map of part of GCHII in the hexagonal crystal form at 1.54-Å resolution, phased by selenomethionine SAD, showing the region close to the zinc ion. A bound sulfate ion is also present. The map was calculated from SHELXE without further density modification. The zinc ion is shown as a magenta sphere, and the sulfate ion is shown in standard atom colors. b, simulated annealing omit map of GMPCPP bound to GCHII in the tetragonal crystal form. Carbon atoms of GMPCPP are drawn in cyan; residues interacting with GMPCPP are drawn in gray. The zinc ion is shown as a magenta sphere and magnesium as a black sphere. c, stereo view of the backbone of GCHII with every 20 residues marked in red. d, ribbon diagram of GCHII with secondary structure marked and showing bound ions as observed in the hexagonal crystal form. GCHII is shown in rainbow colors from blue at the N terminus to red at the C terminus. The zinc ion is shown in magenta, and the sulfate ions and sulfonate fragment of HEPES are shown in standard atom colors. e, ribbon diagram of the GCHII dimer with bound GMPCPP in the tetragonal crystal form. The helices of the two subunits are shown in cyan and aquamarine, and the β-sheets are shown in yellow and orange. The zinc ion is colored magenta, and the magnesium ion is black. The bound GMPCPP is shown in standard atom colors.
then follow, giving release of formate (Fig. 3, c and d). By analogy with carboxypeptidase A and carbonic anhydrase a penta-coordinate zinc ion, making interactions with the carbonyl group of the formamide intermediate and with a water molecule, may also be present in GCHII (3, 6, 25, 26). Following the completion of ring opening, release of the product DARP occurs, which, if the putative covalent intermediate is present, would involve hydrolysis of the nucleotide from Arg128. All of the residues postulated to be involved in the GCHII reaction steps are fully conserved in the alignment of sequences of a set of 20 GCHII genes from a representative range of bacterial species (data not shown).

**Comparison of the Active Sites of GCHI and GCHII**—To allow comparison of the active sites of GCHI (3) and GCHII, the two zinc ions together with the zinc ligand bonds were overlapped (Fig. 2b). Although the imidazole moieties of the nucleotide bases and ribose rings (of GTP or GMPCPP) partially overlap in the two enzymes, the triphosphate/triphosphate analogue moieties curve off in opposite directions. Additionally, the nature of surrounding residues and other features of the active sites are generally dissimilar. Thr114 in GCHII and Glu152 in GCHI are in a similar position relative to the guanine rings, interacting via hydrogen bonds to O-6 (Thr114) and N-2/N-6 (Glu152). For GCHII, Arg94 contributes to locating GTP by its guanidinium group lying parallel with the plane of the guanine ring. There is no equivalent interaction in GCHI. The primary role of His112 in GCHI is proposed to be as a proton acceptor from the zinc-bound water to activate it for the guanine ring-opening reaction. It has also been proposed that His112 may assist guanine ring opening via a transient Schiff base by protonating the ribose O-4 (27). There is no equivalent ribose O-4-histidine interaction in GCHII; rather Arg94 hydrogen-bonds to O-4. Although Tyr105 (GCHII) and His179 (GCHI) overlap, they do not have exactly the same roles in the two enzymes. Tyr105 interacts with the water linked to the zinc ion, and in this respect, it has a function more closely related to His112 in GCHI. However, His179 (GCHI) and Tyr105 (GCHII) may each

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**TABLE ONE**

| Data collection, phasing and refinement statistics for GCH II |
|-------------------------------------------------------------|
| **Data collection**                                        |
| Data set                                                   | Se-Met apo GMPCPP |
| Data collection site                                      | BM14, ESRF In-house |
| Wavelength (Å)                                            | 0.97912 1.5418 |
| Space group                                               | P6_2,2 P4_2,2 |
| Cell dimensions                                           | a, b, c (Å)  α, β, γ (°) |
| a, b, c (Å)                                                | 71.9, 71.9, 128.6 90, 90, 120 |
| α, β, γ (°)                                                | 90, 90, 90 90, 90, 90 |
| Asymmetric unit (molecules)                               | 1 2 |
| Resolution (Å)                                            | 30.0–1.54 (1.60–1.54)_A 30.0–2.60 (2.69–2.60) |
| Rmerge ^b                                                  | 0.044 (0.245) 0.078 (0.54) |
| I/σ                                                       | 76.4 (2.3) 28.5 (3.0) |
| Completeness (%)                                          | 89.4 (26.5) 99.8 (99.6) |
| Redundancy                                                | 17.2 (1.6) 6.6 (6.1) |
| SAD phasing in SHELXE                                     | 0.553 |
| Map contrast                                              | 0.947 |
| Pseudo-free correlation coefficient                       | 79.8% |
| **Refinement**                                            |
| Resolution (Å)                                            | 30.0–1.54 30.0–2.60 |
| No. reflections                                           | 27331 15244 |
| R_rms/R_free _c                                              | 0.193/0.213 0.209/0.260 |
| No. of atoms                                              | 1324 2656 |
| **Protein**                                               | 1324 |
| **Ligand/ion**                                            | 38 |
| **Water**                                                 | 203 |
| **β-factors**                                             | 23.5 |
| **Protein**                                               | 65.2 |
| **Ligand/ion**                                            | 23.6 |
| **Water**                                                 | 115.0 |
| **Root mean square deviations**                           |
| Bond lengths (Å)                                          | 0.007 |
| Bond angles (°)                                           | 1.40 |
| Ramachandran statistics _d                                 |
| % residues most favored                                    | 92.1 |
| % additionally allowed                                     | 7.9 |
| % generously allowed                                       | 0 |
| % disallowed                                              | 0 |

^a Highest resolution shell is shown in parentheses.
^b Rmerge = Σ|I - (I/σ)|/ΣI/σ.
^c R factor = Σ|F_o - F_c|/ΣF_o.
^d As defined by PROCHECK (32).
act as proton donors during the hydration of the respective formamide intermediates, leading to formate release (3). GCHI does not split pyrophosphate from GTP and hence has no equivalent to Arg128 in GCHII; neither does it contain a bound magnesium ion, also required for this reaction.

**DISCUSSION**

Prior to this report, details of the three-dimensional structure of GCHII and the residues involved in substrate binding and the catalytic mechanism were not known. GCHI and GCHII are of similar size, and both contain a zinc ion for guanine ring opening. However, although both GCHI and GCHII are α/β proteins with a core of antiparallel β-sheet, they have different protein folds. Many of the molecular recognition determinants for GTP in GCHI and GCHII are also different. GCHII does, however, show a weak relationship to hydroxynitrile lyase, which does not contain a zinc ion. GCHI and GCHII provide an example of convergent evolution in which a bound zinc is present for a common catalytic function. Thus for both GCHI and GCHII, two water molecules are added to GTP following activation by the zinc, leading to the release of formate via a formamide intermediate. There are a number of differences in the reaction mechanism of ring opening for the two enzymes. For GCHI, His112 is hydrogen-bonded to the water that is in turn linked to the zinc ion, which helps position it for nucleophilic attack on GTP, whereas for GCHII, Tyr105 fulfills this role. It is thought that such hydrogen-bonding interactions play a critical role in catalysis by correctly locating the bound hydroxide, thereby reducing the entropic barrier for nucleophilic attack on the substrate (26). For GCHI a second histidine has been implicated in the mechanism, His179, which has been proposed to act as a proton donor to the N-7 of the formamide intermediate (6). GCHII is notable for the absence of histidine side chains or acidic side chains close to the guanine ring binding region of the active site to act as proton donors/acceptors; rather Tyr105 appears

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**FIGURE 2. Active sites of GTP cyclohydrolases. a,** stereo view of the binding of GMPCPP in the active site of GCHII. Carbon atoms of GMPCPP are drawn in cyan; those of residues interacting with GMPCPP are drawn in orange. The zinc ion is shown in magenta and the magnesium ion in purple. **b,** stereo view of the overlap of the active sites of *E. coli* GCHI and GCHII with bound GTP and GMPCPP, respectively. GCHII and GMPCPP are drawn in standard atom colors, and GCHI and bound GTP are drawn in gray. Residues of GCHII are labeled in red, and those for GCHI are labeled in black.
to be positioned for this role. Regeneration of the nucleophilic metal-activated water can be achieved in some enzymes by proton transfer to an ionizable side chain, sometimes via a hydrogen-bonded solvent network (26). In the case of carbonic anhydrase this "proton-wire" network is essential for the very rapid turnover rate of that enzyme. Although in GCHII there is a set of hydrogen bonds linking the zinc to Glu92, a potential proton acceptor, there appears little requirement for rapid proton transfer, as the rate of turnover is 8 orders of magnitude lower than that for carbonic anhydrase. The crystal structure of GCHII confirms the three zinc ligands as cysteines, as indicated by mutagenesis studies (6). GCHII is the only example in the Protein Data Bank of a catalytic zinc that is chelated by three cysteine residues, as (in addition to the water ligand) at least one of the three amino acid side-chain ligands is usually histidine, with other interactions provided by cysteine, glutamic acid, or aspartic acid (28).

Other steps in the GCHI and GCHII reactions are different; i.e. GCHII catalyzes release of pyrophosphate (unlike GCHI), whereas GCHI rearranges the ribose and opened guanine groups into a pterin derivative. Previous kinetic data had provided evidence for a covalent intermediate in the GCHII reaction (10). The crystal structures of GCHII reveal the proximity of Arg128 to the α/β-phosphonate groups of GMPcPP, pointing to the role of this side chain as the nucleophile that attacks the α-phosphorus of GTP. Arg128 is thus positioned to form the putative covalent intermediate, an unusual role in enzymes involving guanylation or adenylation. In the case of RNA/DNA ligases and RNA capping enzymes, lysine invariably forms the covalent link to the nucleotide (29). There are however, some parallels between the formation of the putative covalent intermediate involving Arg128 in GCHII and the nucleophilic attack of arginine on the γ-phosphoryl group of ATP in the arginine kinase reaction (30). The arrangement of negatively charged groups on each side of the guanidinium moiety of the arginine substrate in arginine kinase, where salt bridges to two carboxyls are formed, is similar for Arg128 in GCHII, except for the replacement of one carboxyl by the α-phosphate of GTP in the latter case. The early stages of the GCHII reaction, giving pyrophosphate release, appear to be rate-limiting in what is an extremely slow enzyme reaction anyway, and thus arginine may be a non-optimal residue for this role. GCHII is only 90% efficient in converting GTP to DARP with GMP being formed as a by-product. The lack of domain closure in GCHII on binding the substrate analogue may allow access of water to the active site, hence prematurely hydrolyzing the putative guanylyl intermediate and releasing GMP. The question arises as to why a more rapid GCHII turnover rate, together with reduced rate of side reactions, has not evolved. Presumably, as only relatively small amounts of riboflavin coenzymes are required by the bacteria, high flux through the pathway is not necessary, and thus there is little evolutionary pressure to improve efficiency.

The availability of crystal structures of GCHII providing detailed stereochemistry of the active site and substrate analogue binding should stimulate the design of inhibitors that may have potential as antibacterial drugs. A structure-based inhibitor design strategy based on knowledge of the GCHII active site geometry and incorporating appropriate functionality to target the zinc ion, such as thiol or acidic groups, could lead to potent compounds. Such an approach has been highly successful previously in producing tight-binding inhibitors of the zinc-containing angiotensin-converting enzyme, leading to drugs that have been widely used clinically for hypertension (31). Development of potent GCHII inhibitors holds out the prospect for novel antimicrobial agents, which are urgently needed to combat life-threatening drug-resistant bacteria.

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