Structural basis of biopterin-induced inhibition of GTP cyclohydrolase I by GFRP, its feedback regulatory proteins

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Running title: The inhibitory GTPCHI-GFRP complex
SUMMARY

GTP cyclohydrolase I (GTPCHI) is the rate-limiting enzyme involved in the biosynthesis of tetrahydrobiopterin, a key cofactor necessary for nitric oxide synthase, and for the hydroxylases that are involved in the production of catecholamines and serotonin. In animals, the GTPCHI feedback regulatory protein (GFRP) binds GTPCHI to mediate feed-forward activation of GTPCHI activity in the presence of phenylalanine while it induces feedback inhibition of enzyme activity in the presence of biopterin. Here, we report the crystal structure of the biopterin-induced inhibitory complex of GTPCHI and GFRP and compared it with the previously reported phenylalanine-induced stimulatory complex. The structure reveals five biopterin molecules located at each interface between GTPCHI and GFRP. Induced-fitting structural changes by the biopterin binding expand large conformational changes in GTPCHI peptide segments forming the active site, resulting in inhibition of the activity. By locating DOPA-responsive dystonia mutations in the complex structure, we found mutations that may possibly disturb the GFRP-mediated regulation of GTPCHI.
INTRODUCTION

GTP cyclohydrolase I (GTPCHI, E.C. 3.5.4.16), which is a 260-kDa decamer of homologous subunits, catalyzes the conversion of GTP to dihydroleptopterin triphosphate, the first and rate-limiting step involved in the de novo synthesis of tetrahydrobiopterin (BH₄) in animals. BH₄ plays key roles in phenylalanine catabolism and the biosynthesis of catecholamines and serotonin by acting as an essential cofactor for hydroxylases of phenylalanine, tyrosine and tryptophan. In humans, the autosomal recessive and dominant mutations of GTPCHI are known to cause hyperphenylalaninemia with severe neurological disorders and the 3,4-dihydroxyphenylalanine (DOPA)-responsive form of dystonia (DRD), respectively (1-4). BH₄ also plays a crucial role in the nitric oxide signaling as a cofactor for nitric oxide synthase (5, 6). Recently, BH₄ depletion impairing nitric oxide synthesis has been implicated in endothelial dysfunction associated with hypertension, hypercholesterolemia and diabetes mellitus (7-10).

In the presence of the GTPCHI feedback regulatory protein (GFRP), mammalian GTPCHI acts as an allosteric enzyme regulated by two effector molecules, BH₄ and phenylalanine. GFRP, which is a homo-pentamer (50kDa), binds GTPCHI to mediate feedback inhibition by BH₄ and feed-forward stimulation by phenylalanine (11, 12). The feedback inhibition is also induced by dihydrobiopterin (BH₂) as well as BH₄ (13). Gel filtration experiments have suggested that the resulting BH₄-induced inhibitory or phenylalanine-induced stimulatory complex contains two GFRP pentamers and one GTPCHI decamer (14-16).
To date, no GFRP has been found in bacteria and bacterial GTPCHIs exhibit no cooperative effects with respect to enzymatic activity. Bacterial GTPCHI catalyzes the first step in the *de novo* synthesis of folic acid, compared to BH₄ in animals. The *E. coli* GTPCHI molecule consists of two GTPCHI pentamers, which are positioned in a head-to-head manner to form the active decameric enzyme with pseudo 52-point group symmetry (17, 18). This architecture has also been found in the animal GTPCHI structures (19, 20).

Our previous crystal structure of the phenylalanine-induced stimulatory complex of rat GTPCHI and GFRP showed the GTPCHI decamer sandwiched by two GFRP pentamers as (GFRP)₅-(GTPCHI)₁₀-(GFRP)₅ (20). The complex structure also revealed that the active site configuration of the stimulatory complex is similar to that of *E. coli* GTPCHI, which exists in an active form, indicating that GFRP and phenylalanine stabilize the active configuration of rat GTPCHI. In this study, we report a 2.8 Å crystal structure of the BH₂-induced inhibitory complex of rat GTPCHI and GFRP. The structure reveals BH₂-induced structural changes which further our understanding on how this protein complex regulates the enzyme activity at the atomic level and how mutations isolated from patients causes hyperphenylalaninemia and DRD.

**MATERIALS AND METHODS**

*Protein Purification and Crystallization* - Rat GTPCHI and GFRP were expressed in *E. coli* and purified as described previously (16). Crystals of the inhibitory complex were obtained as previously reported (21). For crystallization, BH₂ and dGTP were used rather than BH₄ and GTP since BH₂ is much more stable in solution than BH₄ and dGTP is not.
hydrolyzed by GTPCHI. The effects of these analogues on the formation of the inhibitory complex are comparable to those of BH₄ and GTP, respectively (16).

**Data Collection** - The crystal was transferred into a cryo-protectant solution (14% isopropanol, 0.2 M ammonium sulfate, 10% PEG300, 10% ethylene glycol and 0.1 M MES-Na pH 6), and then rapidly frozen by transferring into liquid nitrogen. During the data collection, the crystal atmosphere was maintained at 100 K with a cryo-stream. The X-ray diffraction data was collected at BL41XU of SPring-8. Measurements of X-ray Absorption Fine Structure (XAFS) spectra of the crystals indicated the presence of zinc ions in the crystals. Following the XAFS measurements, we collected an intensity data set with the wavelength of the K-absorption peak for the zinc atom (λ = 1.2818 Å) to verify the zinc ion positions on an anomalous difference Fourier map. The data was processed with **MOSFLM** (22) followed by **SCALA** (23). The data collection statistics are summarized in Table 1.

**Structure Determination and Model Refinement** - The structure was determined by the molecular replacement (MR) method using **AMoRe** (24) with the GTPCHI decamer structure from the stimulatory complex (20) as a search model. The pseudo D₅ symmetry of the arrangement of ten GTPCHI subunits was identified in solutions of a rotation function calculated with the resolution range of 10-5 Å and an integration vector of 40 Å. The model was refined with **CNS** (25) using rigid-body refinement and simulated annealing techniques under the NCS restraint. Clear electron densities for two GFRP pentamers were found in the map. The model was further refined by rounds of manual rebuilding with **O** (26) and simulated annealing. The electron densities of the BH₂ molecules were found at the interfaces of GTPCHI and GFRP in an initial Fₒ-Fₑ map. The refinement statistics are presented in Table 1. The structure consists of one GTPCH decamer (residues 48-241 for
each subunit), two GFRP pentamers (residues 1-84 for each subunit), ten BH$_2$ molecules, ten zinc ions, ten sodium ions and ten triphosphate parts of dGTP. The N-terminal region (residues 12-48) of all GTPCHI subunits are disordered as observed in those of the stimulatory complex. Tentative sodium ions were found inside the loop β1-β2 (residues 9-14) of GFRP. In the stimulatory complex, these sites were occupied by potassium ions. Molecular diagrams were prepared by MOLSCRIPT (27), RASTER3D (28) and GRASP (29).
RESULTS

Overall Structure of the Inhibitory Complex - The inhibitory complex consists of one GTPCHI decamer (residues 48-241 for each subunit) and two GFRP pentamers (residues 1-84 for each subunit) (Fig. 1A). As in the previous GTPCHI structures, the GTPCHI monomer in our complex consists of a \( \alpha \)-helical domain (\( \alpha_a-\alpha_d \)) and a \( \alpha+\beta \) domain formed by five \( \alpha \) helices (\( \alpha_a-\alpha_i \)) and four \( \beta \) strands (\( \beta_1-\beta_4 \)) (Fig. 2A). These \( \beta \) strands comprise a 20-stranded antiparallel \( \beta \)-barrel bearing a pseudo 5-fold axis in the GTPCHI pentamer. The GFRP monomer forms a (\( \alpha+\beta \)) structure with a \( \beta\alpha\beta\alpha\beta \) topology. Five GFRP monomers form a homo-oligomeric 5-fold \( \beta \)-propeller structure, where each monomer represents a blade of the propeller in the classical \( \beta \)-propeller protein. Two GFRP pentamers are docked at the top and bottom molecular surfaces of the torus-shaped GTPCHI decamer, sharing the same 5-fold axis. This sandwich structure, \((\text{GFRP})_5-(\text{GTPCHI})_{10}-(\text{GFRP})_5\), is essentially similar to that of the stimulatory complex (Fig. 1B), though several significant structural differences are found, and will be discussed later.

At the GTPCHI-GFRP interface, three GFRP loops, the loop between strands \( \beta_1 \) and \( \beta_2 \) (loop \( \beta_1-\beta_2 \), residues 9-16), loop \( \beta_3-\beta_4 \) (residues 38-45) and loop \( \beta_5-\beta_6 \) (residues 73-75), are projected toward GTPCHI and make contact with GTPCH. Loop \( \beta_3-\beta_4 \) contacts GTPCHI at the outer region of the interfaces between GFRP and GTPCHI, while loops \( \beta_1-\beta_2 \) and \( \beta_5-\beta_6 \) make contact with GTPCHI at the innermost region of the interfaces. As in the stimulatory complex (20), ten zinc ions (red balls in Fig. 1) were found at the active sites in the inhibitory complex and were verified on an anomalous difference
Fourier map calculated with the data set collected with a wavelength for the K-absorption edge of zinc atoms.

**Biopterin-binding Site** - Biopterin is an essential ligand for the formation of the inhibitory complex (11, 16). Ten BH$_2$ molecules were found at each of the GTPCHI-GFRP interfaces (Fig. 1A). This observed stoichiometry is consistent with the results from previous studies that dealt with the binding of BH$_4$ to the protein complex (30). Like the phenylalanine molecules in the stimulatory complex (20), the BH$_2$ molecules in our complex are completely buried inside the interfaces. The total buried accessible surface area of each GFRP-GTPCHI interface, including the five trapped BH$_2$ molecules, was increased to 7,322 Å$^2$, which is significantly larger than that area (4,546 Å$^2$) without the BH$_2$ molecules. Thus, BH$_2$ binding enhances the association of GFRP with GTPCHI by occupying the spaces at the interfaces to increase the contact area.

Interestingly, the BH$_2$-binding sites are located at the molecular surface of GTPCHI rather than GFRP. The bound BH$_2$ molecules make extensive interactions with GTPCHI, resulting conformational changes in GTPCHI. This is in contrast to the phenylalanine-binding sites found in the stimulatory complex, where the GFRP pentamer forms phenylalanine-binding cavities to accommodate the phenyl group (Fig. 1B). Each BH$_2$-binding pocket is formed by a cleft between two neighbouring GTPCHI subunits, designated as $A$ and $B$ (Fig. 2B). These consist of the C-terminal region (residues 220-234) spanning β-strand 4, α-helices h and I from both subunits, and flanking regions of β-strands 1 and 2 (residues 118-120 and 148-150, respectively) from subunit $B$. The pterin ring of BH$_2$ is located within the cleft and forms hydrogen bonds between the pyrimidine part of
the ring and Glu-234(B) located at the bottom of the cleft. These interactions are reminiscent of those between a heme carboxylate and the pyrimidine part of the pterin in the NOS pterin binding site (6). All hydrogen-donor and -acceptor atoms of the pterin ring participate in hydrogen bonding interactions (Fig. 2C). Two water molecules were found to mediate the interactions between BH$_2$ and GTPCHI. Asp-118(B) and Arg-226(A) form direct hydrogen bonds with BH$_2$ as well as a water (WAT2)-mediated hydrogen bond. Arg-232(A), which interacts with Glu-224(B), forms direct bonds with BH$_2$. Arg-232(A) and Asp-228(B) trap another water molecule (WAT1), which mediates two hydrogen bonds to BH$_2$. The main chain of Ser-219(A) and the side chain of Thr-231(B) participate in direct hydrogen bonds to BH$_2$. It is noteworthy that the pyrimidine part of the pterin ring is associated with eight hydrogen bonds. This extensive interaction is consistent with the fact that inhibitory complex formation is induced by 2,4-diamino-6-hydroxypyrimidine and analogs that contain a pyrimidine structure like that found in biopterin (13).

**Structural Changes in GFRP** - Compared with the GFRP structure in the stimulatory complex, no global structural change was found in the monomer and pentamer of GFRP in the inhibitory complex. We obtained small root mean square (r.m.s.) deviations of C$_\alpha$-carbon atoms (residues 2-83) between the GFRP structures of the stimulatory and inhibitory complexes, 0.64 Å for the pentamers and 0.42 Å for the monomers (Fig. 3A and B). However, a local structural change was found in loop β3-β4 (residues 38-44), which interacts with GTPCHI residues located close to the biopterin-binding site (Fig. 1A). The aforementioned structural changes at the interface were correlated with an observed rotational movement of GFRP against GTPCHI. By fitting GFRP pentamers in the
inhibitory and stimulatory complexes, we found that GTPCHI rotates approximately -4.0°
around the 5-fold axis of the torus structure (Fig. 3D). Moreover, each GTPCHI monomer
rotates by 3.3° around the second axis nearly perpendicular to the 5-fold axis.

*Structural Changes in GTPCHI and Inhibitory mechanisms* - In contrast to the limited
structural changes in GFRP, GTPCHI in the inhibitory and stimulatory complexes
exhibited several structural changes (Fig. 3C). In particular, large structural changes were
localized at the loop regions between secondary structure elements, and involved contacts
with neighboring subunits and the active site. The most prominent structural changes in
GTPCHI were found in three loop regions represented by loop $\alpha_d$-$\beta_1$ (residues 106-121),
loop $\beta_2$-$\alpha_e$ (153-161) and loop $\alpha_g$-$\beta_4$ (204-213). All these regions participate in forming the
active site. The most significant structural changes were found in loop $\alpha_d$-$\beta_1$, which
exhibited positional shifts greater than 5 Å. These changes are directly linked to the
structural changes in the bipterin-binding site. In the inhibitory complex, Asp-118 from
subunit $B$ shifted position and orientation to interact with both Arg-226 from subunit $A$ and
the bound BH$_2$ molecule (Fig. 4A). This shift seems to trigger large conformational
changes in residues 110-115 of the loop region containing Phe-113 and Glu-115 (Fig. 4B).
We then superimposed the GTP molecule found in the crystal structure of *E. coli* GTPCHI
and GTP (18) with our GTPCHI-GFRP complexes. It was found that the side chains of
Phe-113 and Glu-115 in the stimulatory complex were projected toward the active site to
cover the guanine ring of the GTP bound at the active site (Fig. 4B, red, 4D, Right). In the
inhibitory complex, however, Phe-113 and Glu-115 were flipped away from the active site
(Fig. 4B, green), resulting in an open state of the active site (Fig. 4D, Left). This peptide
conformation was stabilized by the intra-chain salt-bridge between Asp-114 and His-117. The open state of the active site could be one of the reasons why GTPCHI activity is inhibited in the inhibitory complex, since it is likely that the guanine base of the GTP substrate could not be positioned in the correct orientation in the active site. In the stimulatory complex, Glu-115 is hydrogen bonded to Arg-176 from subunit A (Fig. 4B) and the phenol ring of Phe-113 is stacked on the imidazole ring of His-134 from subunit A, which may contribute to stabilizing the conformation of the active histidine residue (Fig. 4C). These interactions are lost in the inhibitory complex.

The structural changes in loop $\alpha_d\beta_1$ directly affect the structural changes in loop $\beta_2\alpha_e$ of the same subunit by direct main chain-main chain interactions. Among the residues in loop $\beta_2\alpha_e$, Leu-156 is conserved in *E. coli* (Leu-134) and mammalian GTPCHIs (Fig. 2A) and has been shown to be essential for catalytic activity (18) by participating in the formation of a hydrophobic environment in the inner wall of the guanine-binding pocket. In the inhibitory complex, Leu-156 decreased the depth of the binding pocket (by $\sim$2.6 Å) by shifting toward the active site (Fig. 4C).

Other local structural changes were found in loop $\alpha_g\beta_4$ and loop $\alpha_b\alpha_c$ (Fig. 3C). These two loops, which form part of the active site, are exposed to the solvent region and seem to be flexible. The binding of dGTP may cause structural changes in these loops, which appeared disordered in our complex. We observed electron densities for the triphosphate group of dGTP at the position roughly corresponding to that of GTP in the *E. coli* GTPCHI structure (18), although we failed to trace the rest of the dGTP molecule because of poor electron densities. The triphosphate group of dGTP might bind GTPCHI at
the entrance of the active site, while the sugar-base part of dGTP is disordered at the open active site pocket.

**DISCUSSION**

We have determined the BH₂-mediated complex structure between GTPCHI and GFRP and detailed the structural differences between the inhibitory and stimulatory complexes. In both inhibitory and stimulatory complexes, the allosteric effector molecules, biopterin and phenylalanine, are located at the interface between GTPCHI and GFRP while the biopterin-binding pocket is mainly formed by GTPCHI but the phenylalanine-binding pocket is formed by GFRP. The biopterin-binding site of GTPCHI displays no apparent similarity to those of biopterin-required enzymes such as phenylalanine hydroxylase. In our complex, most hydrogen bonds between BH₂ and protein are mediated by the side chains, while those in human phenylalanine hydroxylase are mediated by main chains (31).

Extensive interactions between BH₂ and GTPCHI induce several conformational changes in GTPCHI. Among the several distinct changes observed, the structural characteristics apparently relevant to the underlying mechanisms of enzyme inhibition are those occurring between the BH₂-binding site and the active site. Following BH₂ binding, two stretches of peptide of about six amino acid residues that directly connect the BH₂-binding site to the active site undergo major conformational changes, including a rearrangement in the interaction between the residues of each peptide region. Two crucial amino acid residues (Phe-113 and Leu-156) at the active sites undergo particular spatial dislocation in the inhibitory complex and consequently not only affect the precise spatial
arrangements required for catalysis but also lead to an open active-site structure. By locating DRD mutations in the complex structure, we found mutations that are located at the biopterin-binding site or at the interface between GTPCHI and GFRP that may possibly disturb the GFRP-mediated regulation of GTPCHI.

Human and rat GTPCHIs have flexible N-terminal regions consisting of ~60 and ~50 residues, respectively. These regions, which are disordered in both the inhibitory and stimulatory complexes, have been shown to have no significant effect on complex formation or inhibition by GFRP (19). Consistent with this, only one exceptional mutation in the N-terminal region has been isolated from patients with DOPA-responsive dystonia (32), but almost all mutations have been found in the rest of the GTPCHI molecule, which exhibit a high sequence identity (97.5%) between rat and human. Therefore, using our complex structure together with mutations of human GTPCHI from patients with DOPA-responsive dystonia, we were able to focus on differences at the amino acid level that might account for the modulated structure and function of GTPCHI. In the patients with DOPA-responsive dystonia, 30 missense mutations were found (32, 34-36). Figure 5 shows these mutations mapped onto the three-dimensional structure of the GTPCHI monomer. We classified the mutated residues into five groups shown in different colors in the figure. Most of the mutations were located inside the core domain (magenta) and probably impair the tertiary conformation and/or stability of the protein. Two mutated residues (Cys-132 and His-134) corresponded to active residues located in the active site (red). In the domain protruding into and interacting with the other pentameric structure, there are several mutated residues that seem to interfere with the inter-subunit interactions (blue). The above three categories of mutations may function to inactivate the enzyme activity at least
partially, and are consistent with the hypothesis that partial BH₄ deficiency is the cause of DOPA-responsive dystonia.

The other two groups, however, may be involved in a different mechanism associated with the disease. Four residues in cyan in Figure 5 (Arg-232, Gly-192, Gly-194, and Phe-225) are located in the biopterin-binding site. Arg-232 interacts directly with BH₂ and the other three residues may play important roles in forming the binding cleft by contacting residues that directly interact with BH₂. It is possible that the mutations of these residues affect biopterin binding, ultimately abrogating GFRP's role in the feedback inhibition of GTPCHI. This loss in mediating the regulation of GTPCHI would increase the level of BH₄ in an uncontrolled manner. Two residues (Val-182 and Pro-190) (green) are also mutated in the patient population, which are located in the interface between GTPCHI and GFRP. Interestingly, Val-182 interacts with Leu-40 from GFRP both in the inhibitory and stimulatory complexes, while the interaction between Pro-190 and Asn-43 is observed only in the inhibitory complex. Thus, mutations of Pro-190 and Val-182 could also disturb the GFRP-mediated feedback mechanisms of GTPCHI by weakening the interaction between GFRP and GTPCHI in the inhibitory complex or in both complexes, respectively.

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Footnotes

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Abbreviations

The abbreviations used are: GTPCHI, GTP cyclohydrolase I; GFRP, GTP cyclohydrolase I feedback regulatory protein; DOPA, 3,4-dihydroxyphenylalanine; DRD, DOPA-responsive form of dystonia; BH₄, tetrahydrobiopterin; BH₂, dihydrobiopterin; r.m.s., root mean square.
Legends to figures

**FIG. 1. Overall Structure of the inhibitory GTPCHI-GFRP complex.**

The overall structure of the GTPCHI-GFRP inhibitory complex (A) is compared with the stimulatory complex (20) (B). GTPCHI (yellow) and GFRP (brown) are shown as ribbon models. Each GTPCHI decamer contain ten zinc ions (red balls) at the active sites.

(A) The inhibitory GTPCHI-GFRP complex induced by BH$_2$. Space-filled models are BH$_2$ molecules (magenta). GFRP loop β3-β4 (residues 38-44), which protrudes toward GTPCHI, is labelled.

(B) The stimulatory GTPCHI-GFRP complex induced by phenylalanine. Space-filled models are phenylalanines (cyan).

**FIG. 2. BH$_2$ binding in the inhibitory complex.**

(A) Sequence alignment of rat, human and *E. coli* GTPCHI. Identical residues are highlighted in yellow. The secondary structure elements determined in this study are indicated above the sequence; magenta rectangles (α-helices) and grey arrows (β-strands). The cleavage site observed in rat-liver-derived GTPCHI is indicated (CS) (14). The residues that participate in zinc binding are highlighted in blue. Glu-227 involved in the phenylalanine interaction is highlighted in green. The residues located at the active site (green triangles), the GFRP interface in the stimulatory (red triangles) or inhibitory (red bold triangles) complex, and the BH$_2$-binding site (blue triangles) are indicated below the residues.
(B) A stereo close-up-view of the BH2-binding site. The bound BH2 molecule is drawn as a ball-and-stick model. The BH2-binding site is located at the interface of two GTPCHI subunits, chains A (green) and B (cyan). Two water molecules (red balls) involved in BH2 recognition are also shown with labels (WAT1 and WAT2). The electron density of BH2 found in the $F_o-F_c$ map, calculated without all BH2 molecules, is indicated with contouring at 4σ.

(C) Schematic drawing of the intermolecular interactions between BH2 and GTPCHI. The residues from different chains are colored separately as in B. Hydrogen bonds are denoted as thin lines.

**FIG. 3. Structural changes induced by BH2 binding.**

(A) The positional shifts of corresponding Cα carbon atoms of GFRP in the inhibitory complex relative to those in the stimulatory complex (red) and in the free form (green). The distances are plotted against the residue number. The secondary structure elements of GFRP are shown at the top of the figure.

(B) Superimposition of GFRP monomers in the inhibitory complex (green), stimulatory complex (red) and free-form (cyan, ref. 33).

(C) The positional shifts of corresponding Cα carbon atoms of GTPCHI in the inhibitory complex relative to those in the stimulatory complex. The distances are plotted against the residue number.

(D) Superimposition of GTPCHI monomers in the inhibitory (green) and stimulatory (red) complexes obtained by fitting GFRP pentamers in the complexes. The GTPCHI monomers
are viewed along the 5-fold axis. Rotation of GTPCHI monomers by -4.0° around the 5-fold axis is indicated.

FIG. 4. **BH₂-induced structural changes in the active site**

(A) Comparison of the biopterin-binding sites in the inhibitory (*left*) and stimulatory (*right*) complexes. The binding site consists of residues from GTPCHI subunits A (green) and B (yellow). BH₂ is shown in magenta in the inhibitory complex (*left*). Broken lines indicate hydrogen bonds. Two water molecules (red balls) involved in BH₂ recognition are also shown with labels, W1 and W2, which correspond to WAT1 and WAT2 in Figure 2B, respectively.

(B, C) Comparison of the active sites in the stimulatory (red) and the inhibitory (green) complexes. Two pentamers were superimposed by fitting Cα carbon atoms of the inner β-strands forming a β-barrel in the GTPCHI pentamer rings. The GTP molecule from the *E. coli* GTPCHI-GTP structure (PDB: 1A9C) is fitted to the structures. (C) is a 90° rotated view from (B).

(D) The molecular surfaces of the active sites in the inhibitory (*left*) and stimulatory (*right*) complexes. The GTP molecule from the *E. coli* GTPCHI-GTP complex is overlaid.

FIG. 5. **Mutations mapped on the GTPCHI structure.**

Missense mutations identified in DRD patients. Residue numbers were derived from human GTPCHI to rat GTPCHI and mapped onto the rat GTPCHI monomer structure from the inhibitory complex. Mutated residues are colored as follows; residues involved in BH₂ binding (cyan), GFRP binding (green), contacts to the opposite ring (blue), stabilizing the
tertiary structure (magenta) and those located at the active site (red). Location and environment of each mutated residue are summarized in Table 2.
Table 1. Data Collection and Refinement of the GTPCHI-GFRP inhibitory complex.

| Data collection | SPring-8 BL41XU |
|-----------------|------------------|
| X-ray source    | SPring-8 BL41XU  |
| Wavelength (Å)  | 1.2818           |
| \( d_{\text{min}} \) (Å) | 2.8              |
| Number of Reflections (Observed / Unique) | 533,591 / 83,649 |
| Multiplicities | 6.4 (6.4)        |
| Completeness (%) | 99.9 (99.9)     |
| Anomalous completeness (%) | 99.9 (99.9) |
| Space group     | \( P2_1 \)       |
| Unit cell       | \( a=121.7, b=109.7, c=130.3 \) Å, \( \beta = 97.8^\circ \) |
| \( R_{\text{sym}} \) (%) | 8.9 (38.6)       |
| \(<\text{l}> / <\sigma\text{l}>\) | 7.6 (1.9)        |

| Refinement      |                   |
|-----------------|-------------------|
| Resolution range (Å) | 15 – 2.8         |
| \( R / R_{\text{free}} \) (%) | 20.7 / 23.3     |
| No. of non-H Atoms |                   |
| Protein         | 22,100            |
| \( \text{BH}_2 \) / triphosphate | 170 / 130       |
| Water / \( Zn^{2+} \) / Na\(^+\) | 230 / 10 / 10  |
| \( B_{\text{average}} \) (Å\(^2\)) |                  |
| Protein         | 33.4              |
| \( \text{BH}_2 \) / triphosphate | 27.5 / 92.6     |
| Water / \( Zn^{2+} \) / Na\(^+\) | 24.5 / 55.7 / 41.8 |
| R.m.s. deviations |                  |
| Bond length (Å)  | 0.0085            |
| Bond angles (°)  | 1.417             |

\( a \) The values in parenthesis represent statistics of the outer shell (2.94-2.80 Å).

\( b \) \( R_{\text{sym}} = \frac{\sum_{hkl} |I_{\text{mean}} - |I_i|}{\sum_{hkl}|I_i|} \)

\( c \) \( R = \frac{\sum_{i} |F_o| - |F_c|}{\sum_{i} |F_o|} \), where \( F_o \) and \( F_c \) are the observed and calculated structure factor amplitudes, respectively. 5% of the refractions were excluded when the calculating free-\( R \) value.
### Table 2. Characterization of the GTPCHI point mutations in DRD \(^a\).

| Position \(^b\) | Location | Environment |
|---------------|----------|-------------|
| Pro-14 (L)    | -        | not detected due to flexibility |
| Leu-62 (Q)    | helix \(\alpha\beta\) | packs against lower subunit of Ile69, Ser72 & Leu73 |
| Ala-65 (V)    | helix \(\alpha\beta\) | packs against lower subunit of Ala85, Ser68 & Ile69 |
| Leu-70 (P)    | helix \(\alpha\beta\) | packs against same subunit of Pro77, Leu82 & Pro86, lower subunit of Leu136 & Leu170 |
| Gly-74 (A)    | loop \(\alpha\beta-\gamma\) | open to bulk solvent |
| Arg-79 (W/P)  | loop \(\alpha\beta-\gamma\) | interacts with lower subunit of Arg175 & Glu133, same subunit of Glu75 |
| Gly-81 (V)    | loop \(\alpha\beta-\gamma\) | close to His134 (catalytic residue) of lower subunit |
| Met-93 (K)    | helix \(\alpha\gamma\) | packs against same subunit of Met131, Tyr66 & Phe96, lower subunit of Ile69 & Leu73 |
| Gly-99 (D)    | helix \(\alpha\delta\) | packs against same subunit of Glu102, Ile165 & Arg168 |
| Tyr-100 (N)   | helix \(\alpha\delta\) | interacts with same subunit of Leu170 & lower subunit of Glu75 |
| Asp-106 (N)   | loop \(\alpha\delta-\beta1\) | open to bulk solvent |
| Asp-125 (V)   | sheet \(\beta1\) | interacts with same subunit of Arg142 |
| Ile-126 (K)   | sheet \(\beta1\) | packs against same subunit of Val123, Val143, Ile145 & Val163 |
| Cys-132 (W)   | sheet \(\beta1\) | reaction centre & binds zinc ion |
| His-135 (P)   | loop \(\beta1-\beta2\) | reaction centre & binds zinc ion |
| His-144 (P)   | sheet \(\beta2\) | packs against same subunit of Asp125, Val196 & Ile239 |
| Leu-154 (R)   | loop \(\beta3-\alpha\epsilon\) | packs against same subunit of Lys158, Leu159, Ile162 & Leu188 |
| Ser-167 (T)   | helix \(\alpha\epsilon\) | packs against same subunit of Met128, Val163 & Glu164 |
| Arg-169 (S)   | loop \(\alpha\epsilon-\alpha\rho\) | packs against same subunit of Tyr100, Ile165 & Tyr166 |
| Gln-171 (R)   | loop \(\alpha\epsilon-\alpha\rho\) | interacts with same subunit of Tyr166, Arg175 & Gln179 |
| Thr-177 (K)   | helix \(\alpha\rho\) | packs against same subunit of Thr218 & Thr220, neighbor subunit of His117 |
| Val-182 (I)   | helix \(\alpha\rho\) | hydrophobic interactions with GFRP Leu40 |
| Pro-190 (L)   | loop \(\alpha\rho-\beta3\) | interacts with GFRP Asn43 in inhibitory complex |
| Gly-192 (E)   | loop \(\alpha\rho-\beta3\) | packs against same subunit of Leu148, Leu222, Gly223 & Val224 (BH4-binding) |
| Gly194 (R)    | sheet \(\beta3\) | packs against same subunit of Phe225, Met221 (BH4-binding) & Phe235 |
| Met-202 (V)   | helix \(\alpha\gamma\) | packs against same subunit of Val205 & Met206, lower subunit of Met202 |
| Met-212 (T)   | loop \(\alpha\gamma-\beta4\) | attaches with lower subunit of Asp127, Phe129 & Met212 |
| Lys-215 (R)   | sheet \(\beta4\) | open to internal cavity |
| Phe-225 (S)   | helix \(\alpha\delta\) | packs against same subunit of Met120, Leu148, Met221, Thr231 (BH4-binding) & Phe235 |
Arg-232 (W) helix αi interacts with BH₄

- The residues involved in catalysis, BH₄-binding or GFRP-binding are shown in italics.
- The substituted residues found in DRD patients are indicated in parenthesis as one-letter codes.
- In human GTPCHI, this residue is originally Asp.
Figure 1
Figure 2
Figure 3
Structural basis of biopterin-induced inhibition of GTP cyclohydrolase I by GFRP, its feedback regulatory proteins
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