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Biophysical and structural investigation of the regulation of human GTP cyclohydrolase I by its regulatory protein GFRP

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ABSTRACT

GTP Cyclohydrolase I (GCH1) catalyses the conversion of guanosine triphosphate (GTP) to dihydroneopterin triphosphate (H2NTP), the initiating step in the biosynthesis of tetrahydrobiopterin (BH4). BH4 functions as co-factor in neurotransmitter biosynthesis. BH4 homeostasis is a promising target to treat pain disorders in patients. The function of mammalian GCH1s is regulated by a metabolic sensing mechanism involving a regulator protein, GCH1 feedback regulatory protein (GFRP). Dependent on the relative cellular concentrations of effector ligands, BH4 and phenylalanine, GFRP binds GCH1 to form inhibited or activated complexes, respectively. We determined high-resolution structures of the ligand-free and -bound human GFRP and GCH1-GFRP complexes by X-ray crystallography. Highly similar binding modes of the substrate analogue 7-deaza-GTP to active and inhibited GCH1-GFRP complexes confirm a novel, dissociation rate-controlled mechanism of non-competitive inhibition to be at work. Further, analysis of all structures shows that upon binding of the effector molecules, the conformations of GCH1 or GFRP are altered and form highly complementary surfaces triggering a picomolar interaction of GFRP and GCH1 with extremely slow k_{off} values, while GCH1-GFRP complexes rapidly disintegrate in absence of BH4 or phenylalanine. Finally, comparing behavior of full-length and N-terminally truncated GCH1 we conclude that the disordered GCH1 N-terminus does not have impact on complex formation and enzymatic activity. In summary, this comprehensive and methodologically diverse study helps to provide a better understanding of the regulation of GCH1 by GFRP and could thus stimulate research on GCH1 modulating drugs.

1. Introduction

In mammals, the enzyme GTP cyclohydrolase I (E.C. 3.5.4.16, GCH1) is responsible for the catalysis of the conversion of GTP to dihydroneopterin triphosphate, which constitutes the first step in the biosynthesis of tetrahydrobiopterin (BH4) (Blau and Niederwieser, 1985). BH4 is an important cofactor for aromatic amino acid hydroxylases as well as for nitric oxide synthase (Kaufmann, 1993). Therefore, the presence of BH4 indirectly regulates the amount of neurotransmitters like serotonin, catecholamines, melatonin and nitric oxide and plays an important role in the immune or neurological disorders like e.g. 3,4-dihydroxyphenylalanine-responsive dystonia (Suzuki et al., 2003). Defects in BH4 homeostasis have been identified in various disorders ranging from diabetes, hypertension to atherosclerosis, Alzheimer disease and pain disorders (Maita et al., 2002). Enzymes of the BH4 biosynthetic pathway and in particularGCH1 I therefore are interesting targets for the pharmaceutical industry and the development of new drugs restoring physiological BH4 levels would be beneficial for the patients suffering from BH4 mediated disorders.

In a seminal paper by Harada et al. the molecular basis of BH4 homeostasis was uncovered and shown to involve GCH1 and a regulatory protein, now known as GTP-cyclohydrolase-I-feedback-regulatory protein (GFRP), which simultaneously functions as a positive and negative regulator of GCH1 (Harada et al., 1993). The effects of GFRP on GCH1 occur via formation of heteromeric protein complexes between GCH1 and GFRP, which are dependent on the intracellular concentrations of the effector molecules phenylalanine or BH4. Elevated phenylalanine levels lead to stimulation of GCH1 activity, whereas BH4, the end product of the biosynthesis pathway, inhibits GCH1 in a feedback inhibition type mode (Maita et al., 2004). Mammalian GCH1 show cooperative enzymatic activity. Complex formation with GFRP-Phe leads to increased activity at lower substrate concentrations and

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eliminates substrate cooperativity. Conversely, GCH1 alone is allosterically inhibited by BH4. In the presence of GFRP, the inhibitory effect of BH4 is boosted and occurs at lower, physiologically relevant BH4 concentrations. The GCH1-GFRP system can therefore be regarded as a metabolic sensor that establishes BH4 and aromatic amino acid homeostasis.

The human GCH1 sequence comprises 250 amino acids and forms a 270-kDa, D5-symmetric homodecameric functional enzyme complex in solution (Nar et al., 1995; Hatakeyama, 1989). GFRP occurs as a pentamer of 50 kDa (5 × 10 kDa). GCH1-GFRP complexes consist of one GCH1 decamer flanked by two pentameric GFRP complexes. Association is along the particle fivefold axes and the complexes are approximately 370 kDa in size (Yoneyama and Hatakeyama, 1998).

Here, we present enzyme kinetic data and biophysical studies based on surface plasmon resonance (SPR) and thermal shift assays (DSF), which analyze the formation of the complex between human GCH1 and GFRP and the allosteric regulation of GCH1 enzyme activity. Moreover, we present high-resolution crystal structures of GFRP in the presence and absence of the effector molecule phenylalanine and free as well as substrate analog bound human GCH1-GFRP complex structures. The comprehensive analysis allows us to gain a better understanding of the impact and role of GFRPs in the allosteric regulation of GCH1. Finally, we analyzed the effect of the disordered N-terminal 42 amino acids of GCH1 on GFRP complex formation and enzymatic activity to resolve contradicting literature data.

2. Results

2.1. Protein production and enzymatic characterization

Protein expression and purification was performed as described in the literature (Auerbach et al., 2000). We ensured the availability of high quality protein material of both full-length (FL)-hGCH1 and the truncated Δ42-hGCH1 variants (Supplemental Fig. 1) as well as of binding competent hGFRP for comparative functional and biophysical studies (see below). Enzyme activity assays with our recombinant proteins confirmed earlier literature on GCH1 activity, cooperativity and the effects of allosteric regulation via GFRP (Harada et al., 1993). In contrast to these authors, however, we found a slight increase in KM between the allosterically stimulated GCH1 relative to the isolated enzyme (Supplemental Fig. 2A and Supplemental Table 4).

2.2. Compounds used for the crystallographic studies

BH4 and 2,4-diamino-6-hydroxypyrimidine (=DAHP) analogues are known to bind to the allosteric site of GCH1. In the literature, these are described as non-competitive inhibitors (Meyer et al., 2019). DI00613584 was identified as a micromolar allosteric GCH1 inhibitor in an enzymatic assay (IC50 = 18.6 μM). 7-deaza-GTP, a substrate analog inhibitor of GCH1, was employed as a binder to the active site of GCH1.

2.3. Crystal structures of hGCH1-hGFRP complexes

In order to study BH4 or phenylalanine mediated GCH1-GFRP complex formation, we used generated high resolution X-ray structures of the human inhibitory and stimulatory GCH1-GFRP complexes in the absence and presence of active site binders. Furthermore, we determined structures of human GFRP in absence and presence of its effector molecule phenylalanine. Analysis of the crystal structures helps to delineate the role of the effector molecules and the regulatory protein GFRP in the inhibition of GCH1. Table 1 shows an overview of the structures presented here. Data collection and refinement statistics are summarized in Supplemental Fig. 1.

We were able to determine one free and one substrate bound structure of both the inhibitory and the stimulatory human GCH1-GFRP complexes at high resolution. The crystal structures show no substantial differences in both subunit structure and quaternary arrangement when compared to the already published cryo-EM structures of hGCH1 and the available structures of the rat protein complexes (Maita et al., 2002, 2004; Ebenhoch et al., 2020). Consistent with the EM derived structures, we observe a drastic reorganization between stimulatory and inhibitory complexes that involves the compression of the central 5-helix bundle and concomitant reduction of the diameter of the 20-stranded β-barrel in the inhibitory complex. Since this change in quaternary structure was also observed between unliganded and allosterically inhibited GCH1 structures without GFRP, we concluded that the role of GFRP is merely that of a scaffolding protein (Ebenhoch et al., 2020)].

The difference between the respective free and substrate bound states are minimal. While in the unbound state the region comprising amino acids 118–126 (the “F122 loop”) is disordered and has a poorly defined electron density, binding of substrate leads to an ordering of the F122 loop and thus to the closure of the active site. This phenomenon is observed in both stimulatory and inhibitory GCH1-GFRP complexes.

We have recently shown that allosteric inhibition of GCH1 is not caused by steric obstruction leading to a reduction or loss of binding affinity of the substrate GTP to the inhibited enzyme as previously suggested (Maita et al., 2004), but rather a consequence of accelerated substrate binding kinetics (Ebenhoch et al., 2020). Based on STD-NMR and site-directed mutagenesis experiments we showed that substrate can still bind with similar affinity to the inhibited enzyme, but is not converted, because it dissociates before purine ring hydrolysis is initiated.

We show here now for the first time the binding of 7-deaza-GTP, a medium affinity substrate analogue inhibitor of GCH1, to the inactive inhibitory complex. Fig. 1 compares the binding mode of the substrates in the active and inhibited enzyme complexes. Surprisingly but consistent with our previous findings, the binding mode and interaction details of the substrate analogue in both complexes are very similar.

2.4. Crystal structures of hGFRP in presence and absence of phenylalanine

Previous structural work has delivered structural information on rat

Table 1
Summary of obtained X-ray structures. The colors indicated correspond to the colors in all following figures. Crystallographic data statistics are summarized in Supplemental Fig. 1.

name	protein(s)	PDB ID	ligand	resolution
hGCH1+hGFRP + Phe	hGCH1 + hGFRP	7ALC	Phe	1.7 Å
hGCH1+hGFRP + Phe + active	hGCH1 + hGFRP	7ALB	Phe + 7-deaza-GTP	2.0 Å
hGCH1+hGFRP + allosteric	hGCH1 + hGFRP	7ALA	DI00613584	1.9 Å
hGCH1+hGFRP + allosteric + active	hGCH1 + hGFRP	7ALQ	BH4 + 7-deaza-GTP	2.2 Å
GFRP	hGFRP	7ACC	--	2.0 Å
GFRP + Phe	hGFRP	7AL9	Phe	1.7 Å

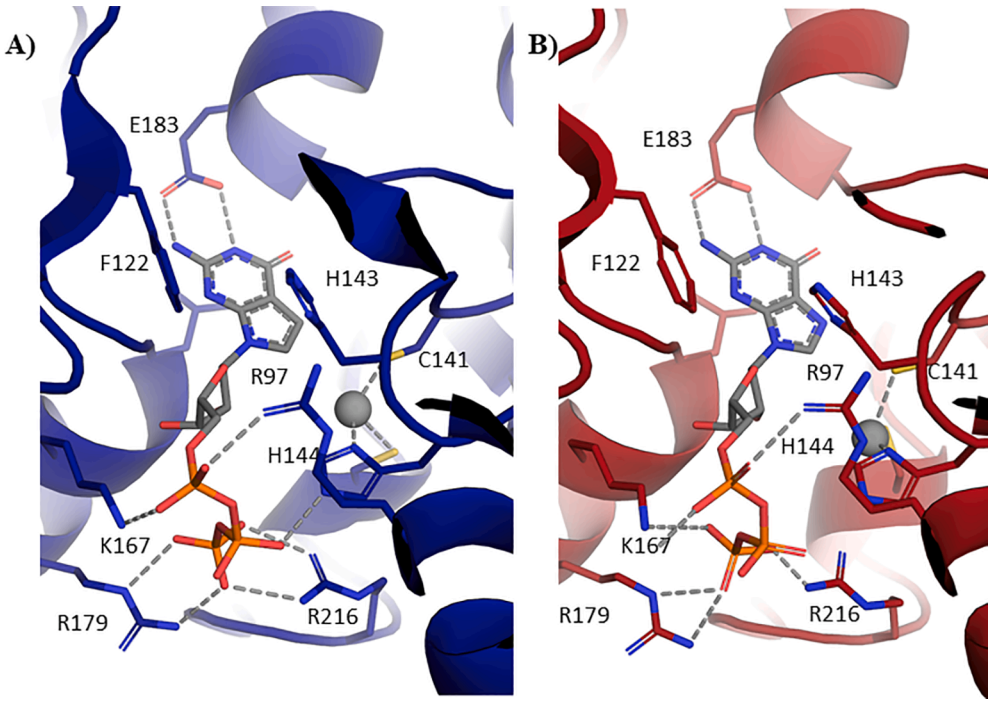

Fig. 1. Substrate binding in the active and inactive GCH1-GFRP complexes. Stick and cartoon representation of 7-deaza GTP (grey carbon atoms) bound to A) the stimulatory complex (blue) and B) to the inhibitory complex (red).

GFRP alone and in complex with rat GCH1 (Maita et al., 2002, 2004) as well as on hGFRP in complex with hGCH1 which we published recently (Ebenhoch et al., 2020). Here we determined the structures of isolated human GFRP (hGFRP) in the presence and absence of phenylalanine. hGFRP assembles in a pentameric ring of five subunits the quaternary structure of which is highly similar to rGFRP and to the hGFRP structures in hGCH1-hGFRP complexes. Likewise, phenylalanine binding does not cause larger rearrangements with rmsds of Cα atoms between the free and the phenylalanine bound hGFRP structures being relatively small – 0.12 Å comparing monomers and 0.23 Å for pentamers. However, local structural differences near the phenylalanine binding site, within residues 9–11 and 74–76, were observed (Fig. 2). Ile10 is situated in hydrophobic cavity in the unbound structure, while it is displaced by the phenyl ring of phenylalanine in the phenylalanine co-structure. The displacement of Ile10 upon binding of phenylalanine leads to a change in the conformation of the loop region comprised of amino acids 9–11 and the induction of a cis-peptide. Upon movement of Ile10, Gln75 is able to form hydrogen bonds to the amino and carboxyl groups of phenylalanine, which are further hydrogen bonding to the Ile10 carbonyl oxygen and Gln75′ NH and Thr76′ carbonyl oxygen of the neighboring chain (Fig. 2B).

We observed binding of an ion, which was previously described as a potassium ion, in both hGFRP structures (Maita et al., 2002, 2004). The potassium coordination by Thr7 and the peptide backbone of Ile10, Arg11 and Val13 does not differ in both structures. Similarly, in inhibitory and stimulatory hGCH1-hGFRP complexes loop 7–13 of hGFRP coordinates a potassium ion in the same manner. We conclude that potassium binding is neither influenced by complexation with GCH1 nor by the binding of effector molecules.

Comparison of the structures of GFRP alone with GFRP the complex with GCH1 shows that GFRP in the stimulatory complex is almost identical to the phenylalanine bound GFRP structure, while non-ligand bound GFRP is very similar to GFRP in the inhibitory complex. This suggests that the introduction of the cis-peptide and loop 9–11 conformation is induced solely by the binding of phenylalanine. GCH1 seems to have little or no influence on GFRP conformation. In general, GFRP appears to be a conformationally very stable protein, showing only minor changes in loop 37–45 upon binding of GCH1. This is in line with the discussed role of GFRP as scaffolding protein (Maita et al., 2002; Yoneyama and Hatakeyama, 1998; Tatham et al., 2009).

2.5. *Formation of the stimulatory complex is mediated by Phe but not by Tyr or Trp*

GCH1́s downstream product BH4 is an essential cofactor for the aromatic amino acid hydroxylases (phenylalanine hydroxylase, tyrosine-3-hydroxylase, and tryptophan-5-hydroxylase). Feed-forward activation of GCH1 via phenylalanine induced GFRP-GCH1 complex formation enhances GCH1́s enzymatic activity and eliminates substrate cooperativity. It has not yet been investigated whether other aromatic amino acids, such as tyrosine or tryptophan, can induce the formation of the stimulatory complex and thus modulate GCH1 activity. Fig. 3A depicts the thermal melting curves for the GCH1 and GFRP as well as GCH1/GFRP mixtures in presence and in absence of BH4. The fusion of the individual protein signals into one single melting point clearly indicates an interaction of the two proteins, which is only observed in the presence of 0.1 mM BH4. The same effect can also be observed in the presence of 20 mM phenylalanine, whereby the melting point of this stimulatory complex indicates a higher thermal stability for the stimulatory compared to the inhibitory complex (Fig. 3B). Strikingly, there seems to be no interaction between GCH1 and GFRP in the presence of 20 mM tyrosine or tryptophan (Fig. 3CD). This suggests that the stimulatory complex is formed exclusively in presence of phenylalanine and that phenylalanine is the only aromatic amino acid capable of modulating GCH1 activity. No complex formation occurs in the absence of the small effector molecules Phe and BH4.

2.6. *GCH1-GFRP complexes form with picomolar affinity, but only in the presence of small molecule effectors*

The DSF data shown above, together with previously published size exclusion chromatography and crystallographic data already showed that the allosteric regulators Phe or BH_4, are essential for the formation of stable stimulatory and inhibitory complexes. SPR data confirm the

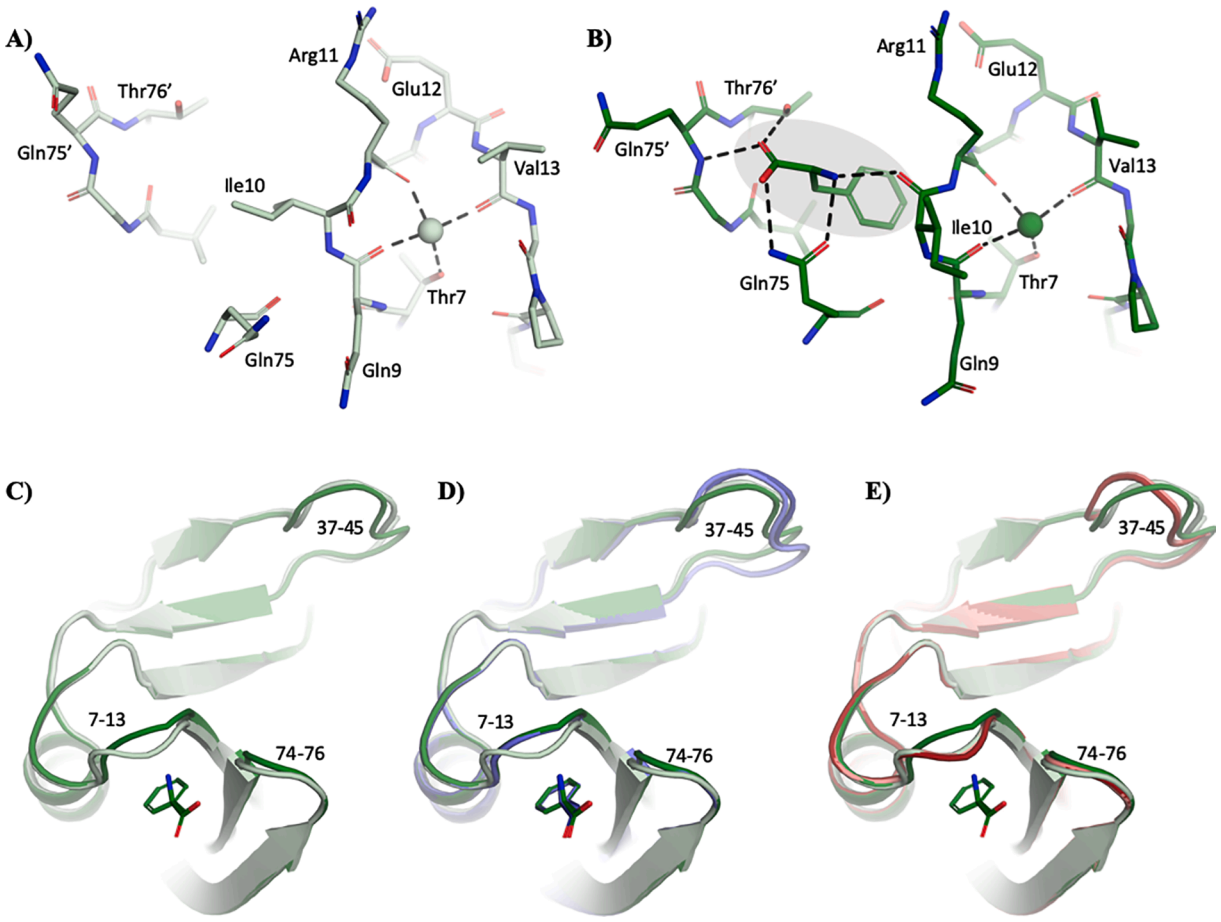

Fig. 2. Conformational rearrangements in free and complexed GFRP. A) Stick representation of the phenylalanine binding site in the GFRP crystal structure (pale green). Ile10 is blocking the binding site. B) The GFRP-Phe crystal structure (green) shows the coordination of phenylalanine in its binding pocket. Superimposition of C) GFRP (light green) and GFRP-Phe, D) GFRP (pale green), GFRP-Phe and GFRP of stimulatory complex (blue) and E) GFRP (pale green), GFRP-Phe and GFRP of inhibitory complex (red).

basic features of GCH1-GFRP complex formation and offer further insights into binding kinetics and protein interaction affinity.

hGCH1 was immobilized on the CM5 biosensor using standard amino coupling chemistry. Formation of the stimulatory complex GCH1-GFRP occurs exclusively in the presence of high concentrations of L-phenylalanine (Fig. 4A). The concentration of Phe that is necessary to obtain the completely saturated formation of free GCH1 decamers with GFPR pentamers was determined in a titration experiment (Fig. 4A), where Phe concentration was varied from 78 μM to 10 mM. No or minor formation of hGCH1-hGFRP complex was observed at low Phe concentration Significant formation of stimulatory complex was observed at concentration of 625 μM and saturation of hGCH1 with hGFRP was reached at 10 mM. The apparent EC50 of complex formation is 2 mM. The dependence of the formation of the stimulatory complex on GFRP concentration was determined by measuring the response to rising GFRP concentrations in the presence of 10 mM phenylalanine (Fig. 5AB). The ternary complexes form with 413 pM affinity. Absence of Phe in the running buffer results in extremely rapid off rates (Fig. 5B). In contrast, we observed very slow dissociation of the complex in the presence of Phe in the running buffer (Fig. 5A).

For the inhibitory complex, a similar picture emerged. We observed binding of GFRP to GCH1 only in the presence of allosteric regulator BH_4. Formation of the ternary GCH1-GFRP-BH4 complex depends on the BH_4 concentration and saturation of GFRP binding to hGCH1 is reached at BH4 concentrations above 500 μM (Fig. 4B). The apparent EC50 of complex formation is 125 μM. Dissociation of the ternary GCH1-GFRP-BH4 complex occurs rapidly in the absence of BH4 (Fig. 5D). The ternary inhibitory complex forms with 108 pM affinity. As expected, the dissociation of the complex is very slow in the presence of BH4 in the running buffer. Dissociation of the complex is about 20-fold faster in comparison with stimulatory complex.

2.7. Small molecule effectors change conformations of GCH1 or GFRP and their surface structures facilitating high affinity molecular recognition

To understand and visualize how the binding of low molecular weight effectors can modulate the affinity of two proteins so drastically as seen in the SPR measurements shown above, we analyzed the protein-protein interfaces of all proteins in the apo, ligated and complex-bound state. When analyzing the rGCH1-rGFRP structures Maita et al. claimed that by binding of phenylalanine to GFRP the total buried surface accessible surface area of each GFRP-GCH1 interface is significantly increased, when compared to the same structure without phenylalanine (Maita et al., 2002). They conclude that the increase the contact area between GFRP and GCH1 by the binding of phenylalanine to GFRP explains the change in affinity. We used PISA (Krissinel and Henrick, 2007) to calculate the interface surface area of the stimulatory and inhibitory human complexes and a hypothetical modelled "stimulatory complex" using non-liganded GFRP instead of GFRP-Phe. The interfaces of the human complexes span an area of 1951 $Å^2$ and 1950 $Å^2$ while the surface area of the hypothetical, unliganded complex is 1806 $Å^2$. We conclude that it is not the change in size of the buried surface area that gives rise to the observed association behavior characterized by a switch from no to picomolar affinity.

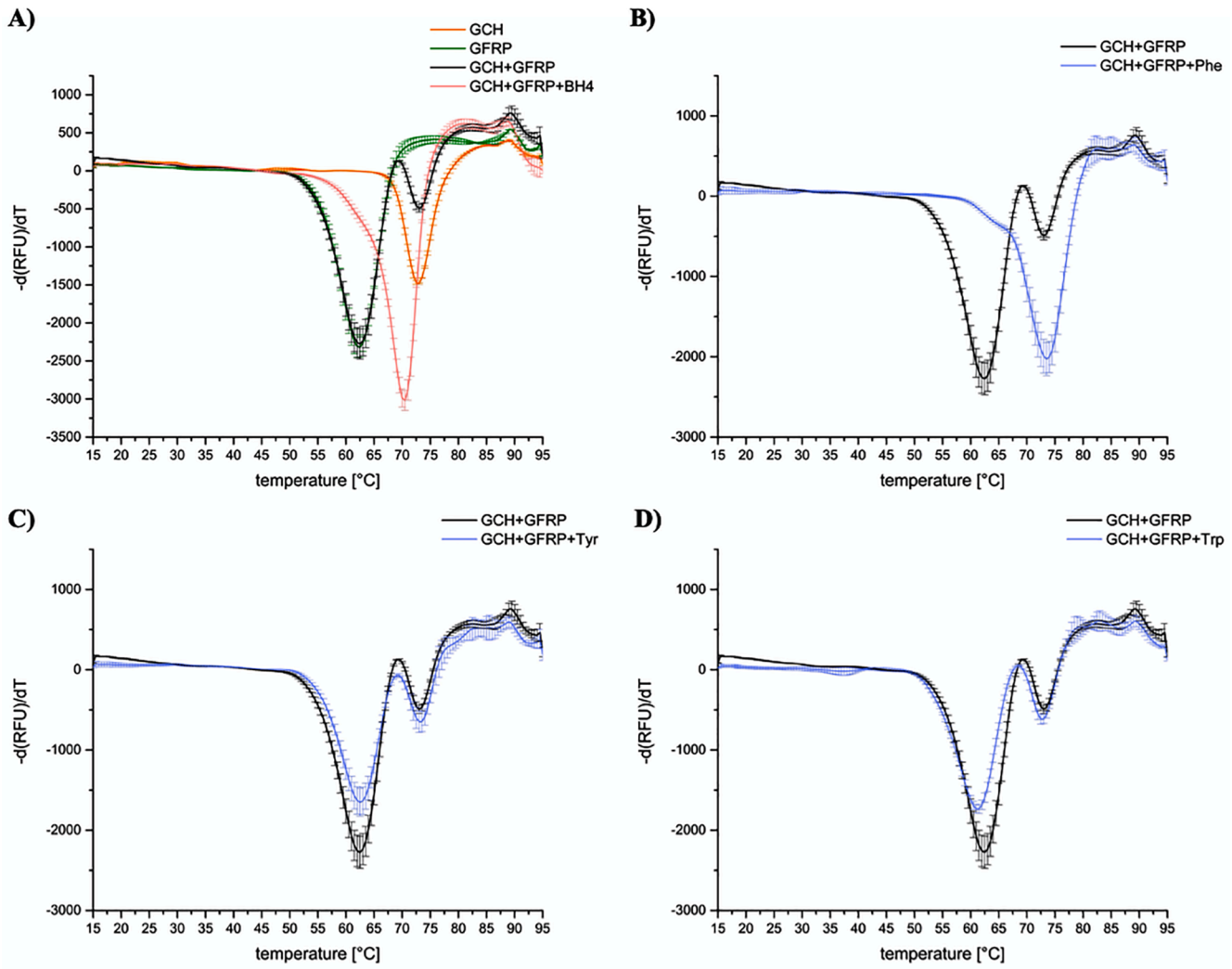

Fig. 3. Thermal shift analysis of the GCH1-GFRP complex formation induced by aromatic amino acids. A) Determination of protein melting points using DSF. T_Ms of GCH1 (orange), GFRP (green), GCH1 + GFRP (black) and GCH1 + GFRP + 0.1 mM BH4 (red) are 73 °C, 62.5 °C, 73 & 62.5 °C and 70.5 °C respectively. B) T_M of 75 °C was measured for the GCH1 + GFRP + 20 mM Phenylalanine (blue). The black melting curve derives from mixture of GCH1 and GFRP in absence of small effector molecules. The same setup is shown for GCH1 and GFRP in presence of C) 20 mM Tyrosine (blue) or D) 20 mM Tryptophan (blue).

We went on to closely analyze the conformational changes observed in GCH1 and GFRP as well as the associated changes in protein surface topology upon small molecule effector binding and any close contacts formed between the small molecules, BH4 bound to GCH1 and Phe bound to GFRP, respectively, and the corresponding two protein binding partners (Fig. 6).

We found that binding of phenylalanine to GFRP induces the same GFRP conformation, which GFRP takes on in the stimulatory complex (Fig. 6BC). Thus, it is clear that this conformational state is caused by phenylalanine alone. In this case, it is mainly changes in Ile10, Gln9 and Gln75 that alter the surface of GFRP. The effector molecule Phe does not directly interact with GCH1. The corresponding modulation of the surface of GFRP-Phe now facilitates the interaction with GCH1. GCH1 hardly undergoes any conformal changes and its GFRP facing surface remains largely in the non-liganded state (Fig. 6EG).

The same applies to the conformational changes in the inhibitory complex. The change in quaternary structure of GCH1 in the GFRP complex is very similar to changes induced by BH4 derivatives alone (Fig. 5FH) (Ebenhoch et al., 2020). There are also no direct interactions between BH4 and GFRP in the inhibitory complex. The BH4 induced large conformational changes in GCH1 alter its surface structure. Conformational rearrangements mainly involve the region 231–239 and 195–200. GFRP remains in the apo state (Fig. 6AD).

In summary, small molecule effectors change conformations of GCH1 or GFRP and their surface structures and electrostatics facilitating high affinity molecular recognition. The effector molecules are not involved in the complex formation but are essential for the induction of the specific protein surface structures that are capable of forming the observed protein–protein interaction. The presence of the effector molecules is therefore a pre-requisite for high affinity binding between GCH1 and GFRP. Without an effector molecule, both proteins are present in the "apo" conformations, which do not present compatible surfaces for mutual binding. The extremely fast dissociation of the complexes in the absence of BH4 or Phe is likewise explained by the structural findings.

2.8. The N-terminus of hGCH1 does not influence hGFRP binding and enzymatic activity

The C-terminal domain of GCH1, which is directly involved in catalysis and is responsible for oligomerization of the protomers, has evolved very conservatively. For instance, the terminal 120 residues exhibit a 60% identity between the E. coli and the human enzymes (Maier et al., 1995; Tazawa et al., 2000; Nar, 2004) suggesting that both the catalytic mechanisms as well as the quaternary structures of GCH1 are very similar across species. In contrast, much greater sequence

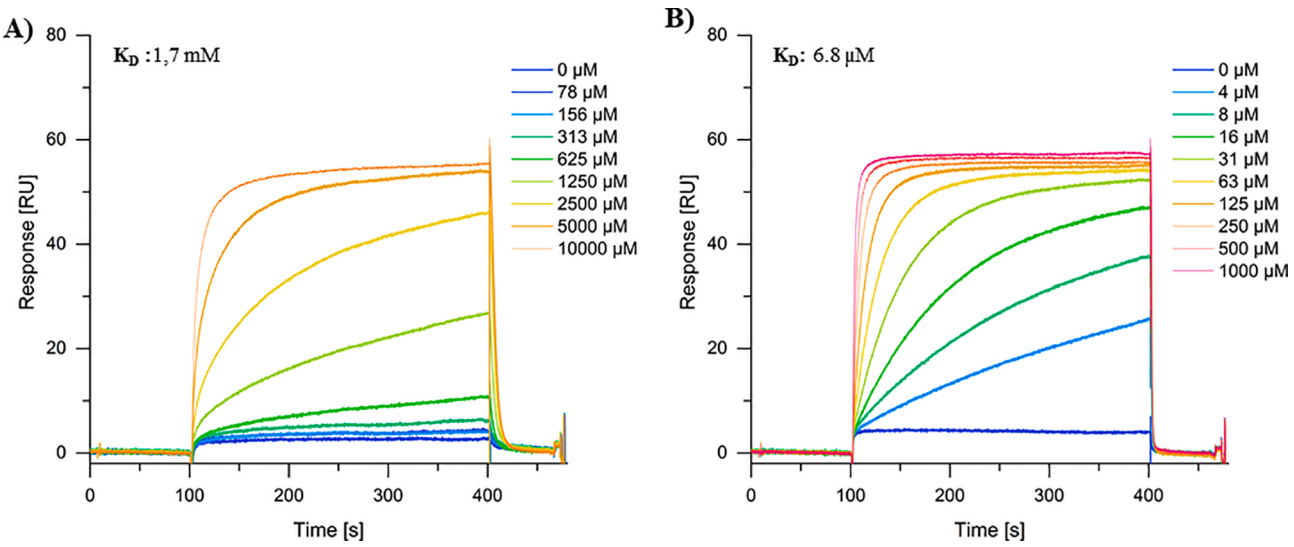

Fig. 4. hGCH1-hGFRP complex formation in dependence of the effector molecules Phenylalanine and BH4. A) Binding of GFRP at 2 µM to the GCH1 in the presence of L-Phe. Concentrations of L-Phe were varied in the range of 10 mM to 78 µM. B) Binding of GFRP at 2 µM to the GCH1 in the presence of BH4. Concentrations of BH4 were analyzed in the range of 1 mM to 7.8 µM. (Supplemental Fig. 4 depicts the steady state affinity fits.)

variability is found at the N-termini of known sequences, indicating that any functional role of the GCH1 N-terminus is unrelated to oligomerisation or catalysis. Further, biochemical and structural data indicate instability of this part of the human and rat GCH1 sequence and its propensity to proteolytic cleavage (Maita et al., 2002, 2004; Auerbach et al., 2000). Analysis of the nature of the sequence suggests that it constitutes a low complexity region and is most probably disordered in the isolated GCH1 structure (Wootton, 1994). In fact, in the published rat complexes the N-terminal domain is not visible in the electron density most probably due to dynamic disorder (Maita et al., 2002). Possible functional roles of the N-termini of mammalian GCH1s include the binding of accessory proteins such as SH3 domains or Aha1 (Swick and Kapatos, 2006).

Previous work has delivered conflicting data about effects of the presence of the N-terminal sequence stretch comprising amino acids 1–42 of hGCH1 on enzymatic activity and GFRP recruitment (Swick and Kapatos, 2006; Higgins and Gross, 2011; Hussein et al., 2015). In order to shed light on the potential role of the N-terminus on these parameters, we first ensured the availability of high quality protein material of both full-length (FL)-hGCH1 and the truncated Δ42-hGCH1 variants (Supplemental Fig. 1) and performed SPR and enzyme activity assays described above for both species (Supplemental Figs. 2 and 3, Supplemental Tables 3 and 4).

In the SPR setup, Δ42-hGCH1 showed the same behavior as the FL protein: without a small molecule effector in the running buffer, the complex disintegrated almost instantly (Supplemental Fig. 1AC). In contrast, the small molecule containing buffer induced a high-affinity binding of the complex partners and almost no dissociation of hGFRP (Supplemental Fig. 1BD). Comparing quantitative binding data, highly similar on- and off-rates as well as dissociation constants are determined for both hGCH1 variants (highest difference of factor 5) and similar trends with respect to BH4 vs. Phe binding. The absence of significant differences in the protein interactions suggest that the presence of the N-terminal domain does not have any impact on GFRP recruitment.

In the enzyme activity assays we found a higher activity for the truncated enzyme both alone and in the stimulated complex similar to previously published data on the rat enzyme (Higgins and Gross, 2011). However, the specific activity was only ~20% higher and the differences in KM and Hill coefficients were similarly small.

In conclusion, our data clearly show that the absence of the hGCH1 N-terminus has only marginal effects on enzyme activity and GFRP association disproving previous literature that insinuated a potential auto-inhibitory function of this domain (Higgins and Gross, 2011) on GCH1 enzymatic activity or a significant role in the formation of GFRP regulated complexes (Hussein et al., 2015; Swick and Kapatos, 2006).

3. Summary and discussion

In this work we present a comprehensive structural, biophysical and enzyme kinetic study of the regulation of GCH1 by its regulatory protein GFRP, which aims to shed light on the mechanism of effector-induced association of GFRP.

High resolution crystal structures of the human stimulatory and inhibitory GCH1-GRFP complexes, each in the liganded and unliganded state, reveal that the substrate analogue 7-deaza-GTP binds to both stimulatory and the inhibitory complexes and adopts an identical binding mode. Structural differences in the catalytically relevant residues are negligible between both complex structures. Upon binding of substrate analogue both complexes undergo the same disorder–order transition in the F122-loop, which transitions from a disordered to a structurally well defined inward facing state and thereby a shields the substrate and locks it in the active site. Based on their rat GCH1-GFRP structures, Maita et al. postulate that the inhibitory regulation is controlled by two defined conformations of the F122 loop. In the inhibitory state, the F122 loop is claimed to have an open conformation, while it is in a closed state in the stimulatory complex (Maita et al., 2004). Furthermore, they observed that in the inhibitory complex the side chain of Leu154/165 (rat and human sequence numbering) is shifted towards the active site, thereby decreasing the depth of the GTP binding pocket. Taking these observations together the authors suggest that the reasons for the reduced GCH1 activity in the inhibitory complex is due to the fact that the guanosine base of the substrate GTP cannot be positioned in the correct orientation in the active site. Our human GCH1-GFRP crystal structures demonstrate that the mode of substrate binding to the inhibitory complex is identical to that to the active enzyme. Furthermore, the topology of the active site pocket and the conformation of the catalytically relevant residues do not differ. This leads us to conclude that the reduced enzyme activity of the inhibitory complex cannot be explained by the inability of binding the substrate. Additionally, both complexes seem to allow for closure of the active site upon substrate binding by conversion of F122 loop from a flexible state to an ordered closed conformation. These observations are consistent with our recently published study which postulates that the substrate affinity of the stimulated and inhibited complexes are very similar and

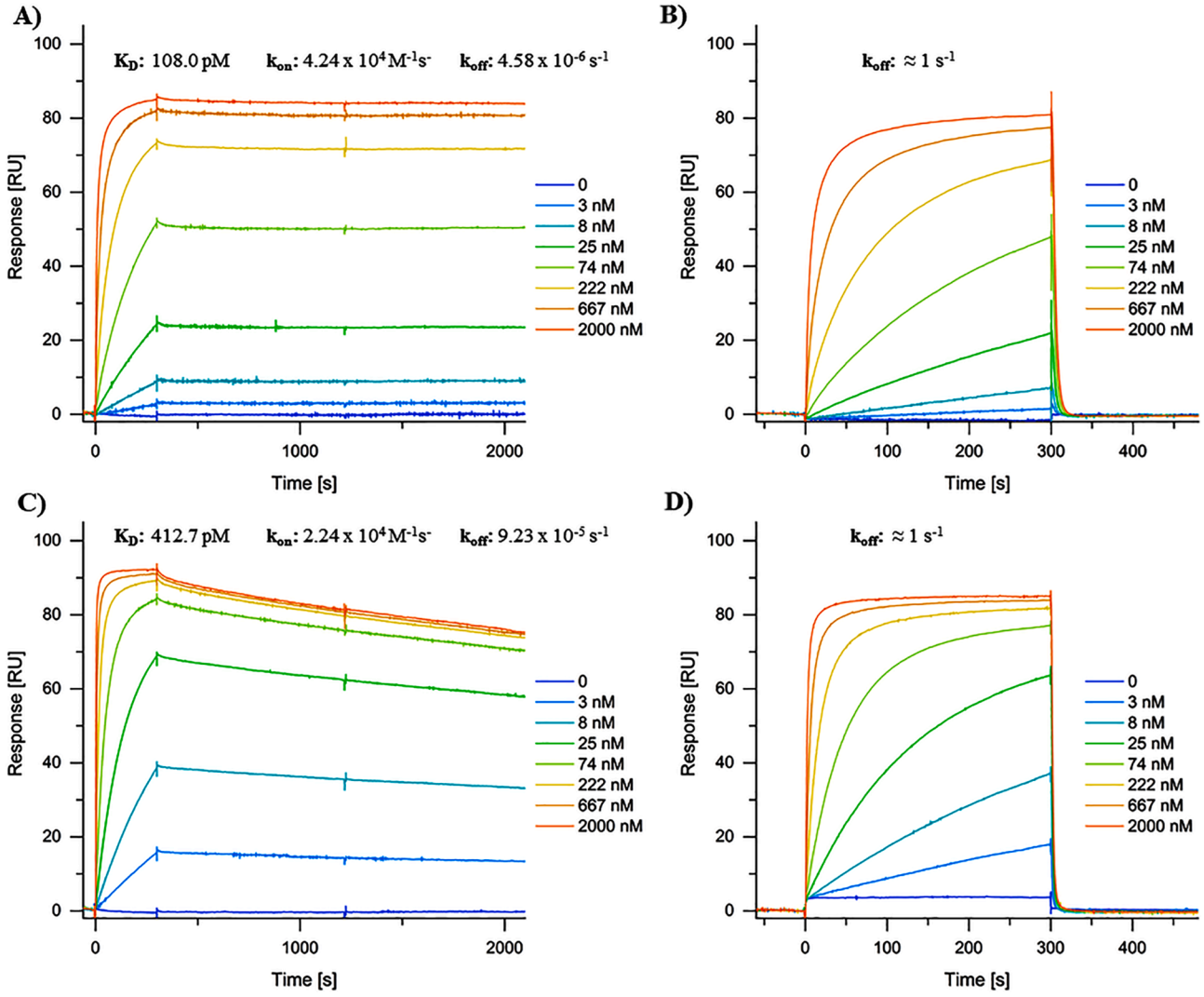

Fig. 5. SPR analysis of the complex formation using different hGFRP concentrations and full-length hGCH1. A) Binding of GFRP to the GCH1 in the presence of 10 mM L-Phe. Binding of GFRP was tested in the concentration range of 2.7 nM to 2000 nM. B) Same experimental setup than in A) but without 10 mM L-Phe in running buffer. C) Binding of GFRP to GCH1 in the presence of 1 mM BH4. Binding of GFRP was tested in the concentration range of 2.7 nM to 2000 nM. D) Same experimental setup than in C) but without 1 mM BH4 in running buffer. (Curves A) and C) were fitted using a 1:1 stoichiometry kinetic model fit, which are depicted in Supplemental Fig. 5).

rather the binding kinetics of the substrate is accelerated in the inhibited enzyme, which reduces the residence time of the substrate and precludes conversion to product (Ebenhoch et al., 2020). This is probably mainly caused by BH4 induced compression and rigidification of the GCH1 central core, which affects the kinetics of the order–disorder transitions of peripheral regions, such as the F122 loop.

BH4 is involved as an essential cofactor for the aromatic amino acid hydroxylases, which catalyze the conversion of L-phenylalanine to L-tyrosine, L-tyrosine to L-DOPA and L-tryptophan to 5-hydroxy-L-tryptophan. So far, it was known that phenylalanine can bind to GFRP, which triggers the formation of the stimulatory complex and thereby leads to a reduction in cooperativity and thereby to an increase in the activity of GCH1. It was unknown whether other aromatic amino acids like tyrosine or tryptophan also address the same feedback mechanism and can bind GFRP, allow formation of GCH1-GFRP complexes and thus stimulate GCH1. In this study we show by DSF experiments that the stimulatory complex is only formed in presence of phenylalanine but not upon addition of tyrosine or tryptophan. Therefore, feedback stimulation seems to be exclusively triggered by phenylalanine.

Small molecule effectors change conformations of GCH1 or GFRP and their surface structures facilitating high affinity molecular recognition. The effector molecules are not directly involved in the complex formation but are essential for the induction of complementary, interlocking surfaces. The presence of the effector molecules is therefore a pre-requisite for high affinity, picomolar binding between GCH1 and GFRP. Without effector molecules, both proteins adopt the “apo” conformations, which do not provide compatible surfaces for mutual binding and therefore lack affinity to each other. A consequence is the extremely fast dissociation of the complexes in the absence of BH4 or Phe, which is most probably driven by the fast dissociation of the weakly binding allosteric effector molecules from each complex and swiftly followed by GFRP dissociation. These observations strengthen the hypothesis of GFRP’s role as a scaffolding protein that functions by either stabilizing the apo or BH4 bound states of GCH1. The stabilization of the inactive state of GCH1 in the presence of GFRP causes an increase of BH4 affinity and a boost of its inhibitory potency. This supports GFRP’s biological role as metabolic sensor and enables rapid and effective modulation of GCH1′s activity. In previous reports, evidence has been provided that GCH1 and GFRP may interact tightly in the absence of small molecule effectors (Hussein, Higgins & Gross). Our data clearly show that this is not the case. It is difficult to speculate what may be the reason for the discrepancy. We also observed complex formation in the

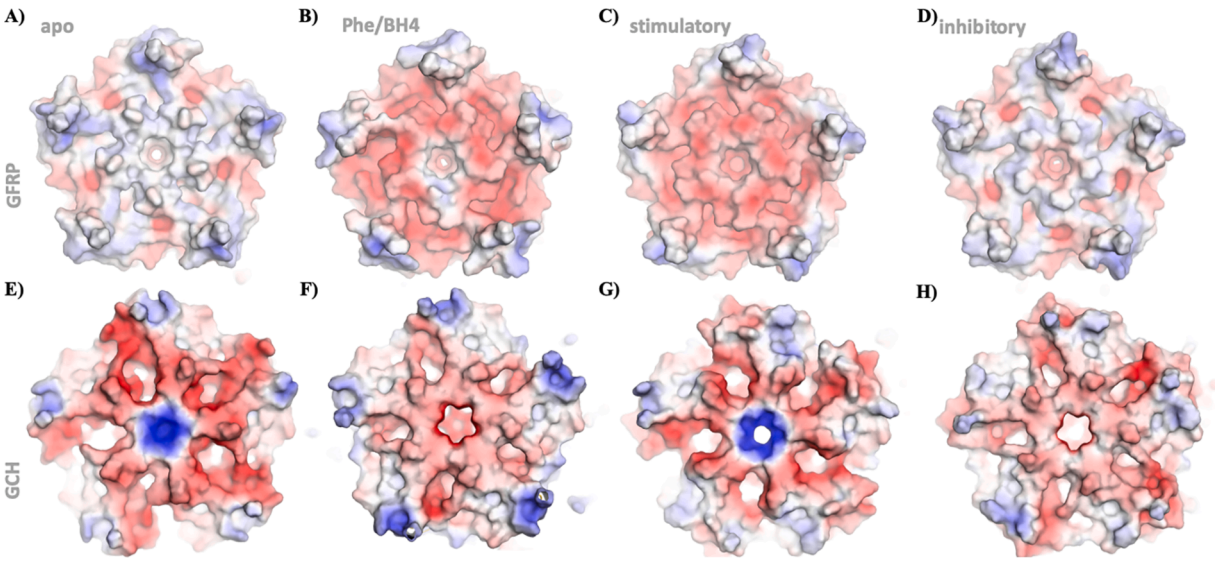

Fig. 6. Surface representation of different conformational states in GCH1 and GFRP colored by electrostatics. A) GFRP. B) GFRP-Phe. C) GFRP-Phe in stimulatory complex. D) GFRP in inhibitory complex. E) GCH1. F) GCH1-allosteric binder. G) GCH1 in stimulatory complex. H) GCH1-BH4 in inhibitory complex.

absence of BH4 and Phe when GTP was present in the buffer system (data not shown). We speculate that presence of GTP in the assays may have caused the production of product H2NTP, which can likewise be able to trigger complex formation, since it features the identical diaminohydroxypyrimidine pharmacophore. It is possible that the literature observations can be traced back to this phenomenon. Further, while Hussein et al. had published the first SPR data on this protein–protein interaction (PPI) and measured KD's in the 10 nM range, our careful analysis of the PPI based on high quality SPR data has shown that the interaction between GCH1 and GFRP is in fact in the 100 pM range and thus 100-fold tighter.

Our study strengthens the hypothesis that GFRP functions as a scaffolding protein that stabilizes the active or inactive conformations of the GCH1 decamer by direct GFRP-GCH1 interactions and by enhancing the binding affinity of allosteric inhibitors. This notion fits well with the relatively small effects of GFPR on enzyme kinetics and the conformational rearrangements. GCH1 alone can be fully inhibited by allosteric inhibitors. In presence of GFRP, the K_i of allosteric inhibitors is decreased by a factor of 10 (Yoneyama et al., 2001). Binding of GFRP does not change the overall conformation of GCH1 significantly as found in the presence of allosteric inhibitors. On the stimulatory branch, from enzyme kinetics we know that GFRP-Phe merely reduces the positive cooperativity of GCH1 and, as a result, slightly stimulates the enzyme's activity in the presence of sub-saturating concentrations of GTP without an effect on V_{max} (Harada et al., 1993; Hatakeyama, 1989). Again, conformational changes of the catalytically active GCH1 decamer between active GCH1 and the stimulatory GCH1-GFRP-Phe complex are small. GFRP seems to help to stabilize the active conformation in each of the ten individual active sites of GCH1, thereby reducing the cooperativity between the active sites and allowing for independent binding of substrate. Although the effects of GFRP on the GCH1 activity appear to be small in terms of enzyme kinetics and structural rearrangements, it has dramatic effects on the response of GCH1 to physiological concentrations of effector molecules BH4 and phenylalanine as originally described by Harada et al. (1993). In fact, continuous variation of tetrahydrobiopterin and phenylalanine concentration in the cellular milieu allows dynamic formation of stimulatory and inhibitory complexes and thus dynamic allosteric regulation of the cellular biopterin homeostasis.

We further provide unequivocal evidence about the role of the GCH1 N-terminus on enzymatic activity as well as in GCH1-GFRP complex formation. While Hussein et al. and Higgins and Gross (2011) suggest that the N-terminal region confers higher activity, induces GFRP binding in the absence of small molecule effectors and may even function as an autoinhibitory element, our data clearly show very little effect and thus suggest that the physiological role of the N-terminal region may indeed be unrelated to the enzymatic function and its allosteric control, but may rather lie in establishing interactions between GCH1 and other binding partners as suggested by a yeast-two-hybrid study (Swick and Kapatos, 2006).

In conclusion, this comprehensive structural and biophysical study characterizes the details of the GCH1-GFRP interaction and elucidates how effector molecules induce conformational changes in order to facilitate picomolar interaction of GCH1 and GFRP. We postulate that GFRP acts as a metabolic sensor, which increases the affinity of BH4, reduces the cooperativity of GCH1 and stabilizes the inhibitory and stimulatory conformation. Further, we present a substrate bound inhibitory complex structure, which strengthens our previously published hypothesis that allosteric inhibition of GCH1 is not driven by reduced substrate affinity, a rearranged active site incapable of performing catalysis, or a difference in substrate shielding, but rather by a novel, dissociation rate controlled mechanism of allosteric, non-competitive inhibition. Reduction of the residence time of the substrate GTP in the active site precludes turnover by dissociation taking place before purine ring hydrolysis is initiated (Ebenhoch et al., 2020).

Since the BH4 pathway is currently perceived as an attractive target to treat pain disorders (Latremoliere and Costigan, 2011; Meyer et al., 2019; Tegeder et al., 2006) with GCH1 being the target with human genetics validation, the understanding of the details of allosteric GCH1 inhibition as well as the methods used here will prove highly useful to identify potential drug candidates that selectively modulate its activity.

4. Methods

4.1. Protein expression and purification - GCH1 constructs

hGCH1 (1–250, N-terminal MBP Tag and TEV cleavage site) and Δ42-hGCH1 (42–250, N-terminal 6xHis Tag and TEV cleavage site) constructs were cloned pET28s or into pET17b vectors, respectively and transformed into BL21(DE3) cells. Cells were grown in LB media until the OD (A600) reached 0.6. The cultures were induced with 1 mM IPTG and grown for an additional 16 h at 4 °C. Bacterial cell pellet was thawed, suspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM DTT, complete protease inhibitor cocktail (Roche), and lysed by sonication. Crude cell lysate was clarified by centrifugation at

45,000*g* for 45 min. Supernatant containing 6xHis-tagged protein purified over Ni-NTA agarose column (Protino Ni-NTA Agarose - Macherey-Nagel). The full length GCH1 protein, possessing a N-terminal MBP-tag, was purified using Amylose resin (New England Biolabs). All proteins were further purified via SEC (Superdex 200 Increase 10/300 GL) using 150 mM NaCl, 25 mM Tris-HCl pH 7.4. The Protein-containing fractions were pooled and concentrated to 7 mg/mL using a centrifugal filtering device (Millipore, 100 kDa molecular-weight cutoffs).

4.2. Protein expression and purification – GFRP

hGFRP (1–84, N-terminal 6xHis Tag and Thrombin cleavage site) constructs were cloned into pET17b and transformed into BL21(DE3) cells. Cells were grown in LB media until the OD (A600) reached 0.6. The cultures were induced with 1 mM IPTG and grown for an additional 16 h at 4 °C. Bacterial cell pellet was thawed, suspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 300 mM NaCl, 1 mM DTT, complete protease inhibitor cocktail (Roche), and lysed by sonication. Crude cell lysate was clarified by centrifugation at 45,000*g* for 45 min. Supernatant containing 6xHis-tagged protein purified over Ni-NTA agarose column (Protino Ni-NTA Agarose - Macherey-Nagel). Further purification was carried out by an anion exchange chromatography (AEXC), after desalting and re-buffering via dialysis. Simultaneously to the dialysis, GFRP His-Tag was cleaved by adding 1.5 U of Thrombin (Sigma T4648, 5 U/\micro L) and incubation at RT while stirring for 24 h. Desalted and His-tag cleaved protein solution was concentrated by using Ultra-15 Centrifugal Filter Unit, 30 kDa cutoff (Amicon). Anion exchange chromatography was performed using HiTrap Q HP, 5 mL (GE Healthcare) column. To reach higher protein purity, SEC was carried out, using HiLoad 26/600 Superdex 200 (GE Healthcare) column with the Äkta Explorer (GE Healthcare).

4.3. Enzyme kinetics

GCH1 specific enzyme activity was determined by spectrometric measuring the concentration of its direct product H2NTP. Synergy™ H1 (BioTek Instruments) and Gen5 2.01 software was used to evaluate H2NTP concentration and the maximal turnover rate (Vmax) within each measuring interval. H2NTP concentration was measured at 330 nm over a measuring period of 2 h using a measuring interval of 2–4 min at 37 °C. Samples were prepared using 2 μM GTPCH-I, varying concentrations of GTP (1–2000 μM) and in absence or presence of 15 mM phenylalanine, 0.1 mM BH4 or 3 μM GFRP. The assay buffer used was 50 mM Tris/HCl, pH 7.5, 100 mM KCl. The specific enzyme activity (A) was calculated using the path length of 0.15 cm and the extinction coefficient $\varepsilon 300nm = 6300\ M{-}1\ cm\text{-}1$ of H2NTP. The resulted specific activity values were plotted against increasing substrate concentration ([S]) and fitted by means of Origin software using the Hill equation which was additionally used to determine the final Vmax values, Hill coefficients (n) and Michaelis-Menten constants (Km). Statistical analysis was performed using the Welch's *t*-test. All enzymatic data was at least measured in triplicates (n = 3).

4.4. Protein crystallization

For crystallization of Δ42-hGCH1 or Δ42-hGCH1-hGFRP complexes, the protein buffer was exchanged by SEC (Superdex™ 200 increase 10/300 GL, GE Healthcare) using 100 mM sodium phosphate buffer pH 5.5 and concentrated up to 6 mg/ml using Amicon centrifugal filters (50 kDa cutoff). All crystals were obtained by sitting drop vapor diffusion using 96-well 3-drop SWISSCI plates (MolecularDimensions). The Protein was mixed in a 1:1 (300 + 300 nl) with reservoir solution and was equilibrated against the reservoir. All crystallization trails were setup using the Mosquito pipetting robot system (TTP-labtech).

hGFRP apo crystals were obtained from a reservoir solution containing 2.4 M di-Sodium malonate; pH 7.0 (JCSG F9). For the complexation with small molecules, 1 mM 7-deaza-GTP (TriLink; N-1044-10), 20 mM Phenylalanine or 1 mM DI00613584 was added to hGCH1-hGFRP (6 mg/ml) or hGFRP (10 mg/ml) prior crystallization. GFRP-Phenylalanineco-crystals were obtained from a reservoir solution containing 0.1 M NaCit pH 5.0; 20% w/v PEG 8000 (Proplex E11). The hGCH1-hGFRP-DI00613584 co-crystal was obtained from GOL-P4K; 0.1 M MB3 pH 8.5; 0.12 M Monosaccharides (Morpheus F11) and the hGCH1-hGFRP-BH4-7-deaza-GTP crystal from 0.2 M Sodium malonate pH 7.0; 20% w/v PEG3350 (Index H03). hGCH1-hGFRP-Phe crystals grew in 2 M NaCl; 0.1 M NaCit pH 6.0 and hGCH1-hGFRP-Phe-7-deaza-GTP crystals grew in 25% MPD 0.2; NH4 Acet; 0.1 M Bis-Tris pH 5.5. Crystals grew at 20 °C after 2–16 days. All crystals, except those grown in Morpheus screens, were flash frozen in liquid nitrogen and cryo protected using 28% Glycerol.

4.5. X-ray data collection, processing and refinement

X-ray diffraction data were collected at the Swiss Light Source (SLS; Villigen, Switzerland) at the PXIII and PXI beamline, and processed with the autoPROC pipeline (Vonrhein et al., 2011) using the XDS package (Kabsch, 2010). Resolution cutoffs were calculated using STARANISO (Tickle et al., 2018). Data processing statistics are listed in Supplemental Tables 1 & 2. The models of GFRP-GCH1 and GFRP were manually fitted using Coot (Emsley et al., 2010) and the resulting model was improved by iterative rounds of manual rebuilding and refinement with autoBuster (Bricogne, 2017). The phases were obtained by molecular replacement (Phaser-MR (Bunkóczi et al., 2013)) using the hGCH1 structure (1FB1), rGCH1-rGFRP complexes (1WPL, 1IS8) or rGFRP (1JG5) as search models. The final models of all crystal structures and the structure factors have been deposited in the PDB (accession code 7ACC, 7AL9, 7ALA, 7ALB, 7ALC, and 7ALQ).

4.6. Surface plasmon resonance

Kinetic and thermodynamic characterization of interaction and allosteric regulation of hGCH1 with its regulatory protein hGFRP was performed on the SPR instrument Biacore T100 (GE Healthcare).

hGCH1 was immobilized using standard amino coupling chemistry on the CM5 biosensor. HEPES buffer (HBS-N, pH 7.2) and 10 mM acetate buffer pH 5.5 supplemented with 1 μM $ZnCl_2$ were used as a running and a coupling buffer, respectively. Activated and deactivated flow cell served as the reference flow channel.

Binding studies were performed in Tris buffer (50 mM Tris, 200 mM NaCl, 0.01% (v/v) Tween 20, pH 7.2). Analysis buffer supplemented with 1 mM BH_4 or 10 mM Phe was used to study binding of stimulatory and inhibitory complexes, respectively. Binding analysis was performed at 25 °C and the flow rate of 50 μL min^{-1}. Every single concentration was analyzed in duplicate and analyzed samples were diluted with factor 2 in the appropriate running buffer. Regeneration of the active surface was performed in the analysis buffer without any allosteric regulator.

4.7. Differential scanning fluorimetry DSF

The Tm of proteins and protein complexes was determined by DSF using the Thermofluor Bio-Rad CFX384 and the Bio-Rad CFX Manager software. 10 μM protein solution was equipped with 5 × SYPRO™ Orange Protein Gel Stain (Invitrogen™) reaching a total volume of 10 μL. Effector molecules and GFRP were added in different concentrations to test their influence on thermal stability. 384 well plates were heated up using a temperature ramp from 15 to 95 °C in 1 °C/min steps.

Declaration of Competing Interest

The authors declare that they were employees of Boehringer Ingelheim at the time of this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsb.2020.107691.

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