GTP Cyclohydrolase I Inhibition by the Prototypic Inhibitor 2,4-Diamino-6-Hydroxypyrimidine

MECHANISMS AND UNANTICIPATED ROLE OF GTP CYCLOHYDROLASE I FEEDBACK REGULATORY PROTEIN

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Linjun Xie‡, Joseph A. Smith‡, and Steven S. Gross‡§¶¶

From the ‡Department of Pharmacology and §Program in Biochemistry and Structural Biology, Cornell University Medical College, New York, New York 10021

2,4-Diamino-6-hydroxypyrimidine (DAHP) is considered to be a selective and direct-acting inhibitor of GTP cyclohydrolase I (GTPCH), the first and rate-limiting enzyme in the pathway for synthesis of tetrahydrobiopterin (BH4). Accordingly, DAHP has been widely employed to distinguish whether de novo BH4 synthesis is required in a given biological system. Although it has been assumed that DAHP inhibits GTPCH by direct competition with substrate GTP, this has never been formally demonstrated. In view of apparent structural homology between DAHP and BH4, we questioned whether DAHP may mimic BH4 in its inhibition of GTPCH by an indirect mechanism, involving interaction with a recently cloned 9.5-kDa protein termed GTPCH Feedback Regulatory Protein (GFRP). We show by reverse transcription-polymerase chain reaction that GFRP mRNA is constitutively expressed in rat aortic smooth muscle cells and further induced by treatment with immunostimulants. Moreover, functional GFRP is expressed and immunostimulant-induced BH4 accumulates in sufficient quantity to trigger feedback inhibition of GTPCH. Studies with DAHP reveal that GFRP is also essential to achieve potent inhibition of GTPCH. Indeed, DAHP inhibits GTPCH by dual mechanisms. At a relatively low concentration, DAHP emulates BH4 and engages the GFRP-dependent feedback inhibitory system; at higher concentrations, DAHP competes directly for binding with GTP substrate. This knowledge predicts that DAHP would preferably target GTPCH in tissues with abundant GFRP.

Tetrahydrobiopterin (BH4)\(^1\) functions as an essential cofactor of nitric oxide synthases (NOSs) and aromatic amino acid hydroxylases (1). Because BH4 availability appears to limit the activity of these enzymes, BH4 levels may dictate the rate of production of key cell-signaling molecules, NO, dopamine, norepinephrine, epinephrine, and serotonin. In mammalian cells, de novo synthesis of BH4 originates from GTP via the sequential action of three enzymes; the first and rate-limiting enzyme in this pathway is GTP cyclohydrolase (GTPCH) (2). The importance of understanding GTPCH regulation is highlighted by the knowledge that intracellular BH4 levels are determined by GTPCH activity, and mutations in the GTPCH gene are responsible for severe diseases including 3,4-dehydroxyphenylalanine-responsive dystonia (3) and cases of atypical phenylketonuria (4).

Mammalian GTPCHs are isolated as decamers of 28-kDa subunits. The crystal structure of the enzyme from Escherichia coli (>70% amino acid homology to mouse, rat, and human GTPCH) reveals that GTPCH is a homodecamer, comprised of two pentameric toroids assembled face-to-face (5). Interestingly, the mammalian GTPCHs, but not those from bacteria, are subject to feedback inhibition by BH4. Feedback inhibition by BH4 is mediated by a decrease in \(V_{\text{max}}\) and is noncompetitive with GTP (6). Peroxis some than BH4, particularly dihydro- and tetrahydro- species, also attenuate GTPCH activity via this inhibitory mechanism (7).

Feedback inhibition involves the formation of a ternary complex between BH4, GTPCH, and an auxiliary protein (6) whose cDNA has recently been cloned and found to encode a 9.5-kDa protein, “GFRP” (GTPCH feedback regulatory protein) (8). Northern blot analysis revealed that GFRP mRNA is abundant in liver and kidney, but detectable in all other tissues examined (heart, brain, spleen, lung, skeletal muscle, and testis) (8). Based on the finding that GFRP is a homopentamer, it has been speculated that one GFRP pentamer binds to each of GTPCH’s pentameric surfaces (9). A distinctive feature of GFRP-dependent feedback inhibition is its reversal by elevated levels of Phe (6, 8). In the absence of BH4, it is notable that Phe induces complex formation between GTPCH and GFRP, resulting in a reduced \(K_m\) for GTP and change in kinetic behavior from sigmoidal to hyperbolic (6). Thus a Phe-rich meal can elicit enhanced levels of BH4. Because BH4 is the rate-limiting cofactor of Phe hydroxylase, this mode of GTPCH regulation protects against toxic accumulation of Phe and may thus serve an important cytoprotective function.

Immunostimulant-induced NO synthesis has been shown to be prevented when BH4 synthesis is inhibited in smooth muscle cells (10), endothelial cells (11), fibroblasts (12), glomerular mesangial cells (13), renal proximal tubular epithelium (14), and cardiac myocytes (15). In reports such as the above, investigators have tested for a role of de novo BH4 synthesis in biological systems by taking advantage of the only agent which is generally considered to be a direct-acting and specific inhibitor of GTPCH, 2,4-diamino-6-hydroxypyrimidine (DAHP). Al-
as an internal control and, because they were designed to span an intron, to assess possible contamination with genomic DNA (16). Histone 3.3 primers were as follows: forward 21-mer, 5'-GAAGAGTTGCGGCCCTCTCTGTCAG-3'; reverse 21-mer, 5'-GGCTCCATGTGCTCCCTGGCTGAA-3'. Quantitative PCR analysis of GFRP expression was performed using an optimized primer set and 1 μl of cDNA according to the following schedule: 95 °C for 5 min, 28 cycles of amplification (94 °C, 45 s; 50 °C, 1 min; 72 °C, 1 min), followed by 10 min at 72 °C. Quantitative PCR amplification for histone 3.3 also used 1 μl of cDNA and an identical schedule to that described for GFRP, except that only 27 amplification cycles were performed. PCR products were resolved by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. This resulted in amplification of predicted size products of 603 bp for GFRP and 213 bp for histone 3.3. Confirmation of the identity of the 603-bp product as GFRP cDNA was provided by restriction analysis with each of four enzymes (BamHI, NcoI, PvuII, and Styl) resulting in 2–3 fragments of the predicted sizes. This amplifier was also subcloned into TA3 pCR™ vector (Invitrogen; Carlsbad, CA), transformed into E. coli DH5α, purified, and subjected to dideoxynucleotide sequencing; this revealed 100% nucleotide identity to GFRP previously cloned from rat liver (8). Densitometric analysis of scanned gels was performed using the NIH Image software, version 1.7.

Expression of GTPCH DNA in E. coli and Purification of GTPCH Protein—GTPCH cDNA was obtained from KASMC by RT-PCR, essentially as described previously (17). Primers used were: forward 22-mer, 5'-ATGGAGAAGCCGCGGGGTGTAA-3'; GTPCH reverse 23-mer (created to contain a BamHI enzyme site) 5'-CATGGTGAGAAATAAGCTTGACA-3'. PCR was performed by standard methods using 1 μl of DNA and the following schedule: denaturation, annealing, and elongation at 94°, 55°, and 72 °C for 1 min, 1 min, and 1 min, respectively, for 30 cycles. PCR products were resolved by electrophoresis on a 1% agarose gel and then digested with sticky/blunt-ends using T4 ligase at 16 °C overnight. The ligated product was transformed into competent DH5α E. coli, and clones were selected on LB agar plates with ampicillin. A positive clone was picked and grown in 1 liter of LB medium at 37 °C with shaking at 250 rpm. Bacterial density reached 0.5 at A600. 500 μl isopyropyl-thio-β-D-galactosidase was added to induce fusion protein expression. After 3 h at 37 °C, the bacterial culture medium was centrifuged for 10 min at 4,000 rpm, and the resulting pellet was resuspended in 50 ml of TEND buffer (per liter: 20 ml of 1.0 M Tris-HCl, pH 7.4, 2.0 ml of 0.5 M EDTA, 11.7 g NaCl, and 154 mg of dithiothreitol per liter), containing protease inhibitors (pepsatin 1 μg/ml, leupeptin 1 μg/ml, phenylmethylsulfonyl fluoride 100 μM). Bacterial suspensions underwent two cycles of freezing in liquid N2 and thawing in a 37 °C water bath. Sodium chloride was added to a final concentration 1 M, and bacteria were lysed using a Branson sonicator. Lysates were centrifuged at 15,000 × g for 30 min at 4 °C, and supernatants were applied to a 5 ml column of amylose resin (New England Biolabs). The column was washed with 50 ml of TEND buffer. GTPCH/MBP fusion protein was eluted with 50 ml of 10 mM maltose in TEND buffer. The eluate was concentrated by microfiltration (Amicon, 100 kDa cutoff) and analyzed for purity by SDS-PAGE and Coomassie Blue protein staining. The purified protein yielded a single strong band on SDS-PAGE of the predicted 83 kDa and migrating at 60 kDa. The molecular mass of the purified fusion protein was consistent with the predicted size of 83 kDa. The purified fusion protein was used for the GTPCH assay.

**EXPERIMENTAL PROCEDURES**

**RNA Isolation**—Total RNA was extracted from rat vascular smooth muscle after 0, 2, 4, 8, or 24 h of treatment with a combination of bacterial lipopolysaccharide (LPS, 30 mg/ml) in the presence of desired test agents. Briefly, the cells were treated with 1 μl of 100 mM dNTPs, 1 μl of Moloney murine leukemia virus, SuperScript II RNaseH (Life Technologies) followed by phenol-chloroform extraction and isopropanol precipitation of total RNA. This procedure was rapid and resulted in RNA isolation without detectable genomic DNA contamination.

**RT-PCR Analysis of GFRP Expression**—10 μg of total RNA was added to a reaction tube containing diethyl pyrocarbonate-treated water to give a final volume of 38 μl. 3 μl of random primers (100 ng/μl) were added to each of the reaction tubes, followed by incubation at 65 °C for 5 min and slow cooling to room temperature for annealing of primers. Primer extension was achieved by adding 5 μl of 10× first strand buffer, 1 μl of Ribonuclease Inhibitor (40 units/μl; Ambion, Austin TX), 2 μl of 100 μm dNTPs, 1 μl of Moloney murine leukemia virus, SuperScript II RT (200 units/ml, Life Technologies, Inc.) and incubating for 1 h at 37 °C. Incubates were then heated for 5 min to 95 °C and cooled on ice.

For amplification of GFRP, primers used were: forward 21-mer (created to contain a HindIII site); 5'-GGAGAGTGTGCTCCCTGGCTGAA-3'; GTPCH reverse 23-mer (created to contain an EcoRI site), 5'-AACACTTGTGTATACCTCCATTGGCT-3'. Histone 3.3 primers were used both as an internal control and, because they were designed to span an intron, to assess possible contamination with genomic DNA (16). Histone 3.3 primers were as follows: forward 21-mer, 5'-GAAGAGTTGCGGCCCTCTCTGTCAG-3'; reverse 21-mer, 5'-GGCTCCATGTGCTCCCTGGCTGAA-3'. Quantitative PCR analysis of GFRP expression was performed using an optimized primer set and 1 μl of cDNA according to the following schedule: 95 °C for 5 min, 28 cycles of amplification (94 °C, 45 s; 50 °C, 1 min; 72 °C, 1 min), followed by 10 min at 72 °C. Quantitative PCR amplification for histone 3.3 also used 1 μl of cDNA and an identical schedule to that described for GFRP, except that only 27 amplification cycles were performed. PCR products were resolved by electrophoresis on a 1% agarose gel containing ethidium bromide and visualized by UV-induced fluorescence. This resulted in amplification of predicted size products of 603 bp for GFRP and 213 bp for histone 3.3. Confirmation of the identity of the 603-bp product as GFRP cDNA was provided by restriction analysis with each of four enzymes (BamHI, NcoI, PvuII, and Styl) resulting in 2–3 fragments of the predicted sizes. This amplifier was also subcloned into TA3 pCR™ vector (Invitrogen; Carlsbad, CA), transformed into E. coli DH5α, purified, and subjected to dideoxynucleotide sequencing; this revealed 100% nucleotide identity to GFRP previously cloned from rat liver (8). Densitometric analysis of scanned gels was performed using the NIH Image software, version 1.7.
thoracic aorta explants of Fisher rats and grown as described previously (10). Cells in passage 5–7 were seeded and grown to confluence for measurement of GTPCH activity. For transfection, cells at passage 5–7 were seeded and grown to 50% confluence in 6-well plates. GTPCH cDNA was used as a template for PCR amplification of a cDNA encoding GTPCH from an EcoRV N-terminal histidine residues and containing convenient restriction sites for ligation into the cytomegalovirus-promoter driven mammalian expression vector, pcDNA3 (Invitrogen). Primers for PCR were: GTPCH forward 49-mer (containing a BamHI site and His-Tag sequence), 5’-CGGGAATCCTACAAGTGCGGATCCACGTCG-3’; GTPCH reverse 21-mer (containing an EcoRV site), 5’-CGGGATCCACGATGCACCAACC-3’. Both the PCR product and pcDNA3 plasmid were digested with BamHI and EcoRV, purified, and ligated at 16 °C overnight. The resulting GTPch-pcDNA3 was transformed into DH5α E. coli, and plasmid DNA was purified from a positive clone. NotI was used to restrict the purified plasmid DNA to confirm correct DNA orientation. Transfection of RASMC was performed in 6-well plates using 15 μg GTPCH-pcDNA3 and 52.5 μl of LipofectAMINE reagent (Life Technologies, Inc.) added to 500 μl of Opti-MEM medium (Life Technologies, Inc.) and mixed for 30 min. While mixing, a plate of cells was twice washed with Opti-MEM and incubated with an additional 5 ml of Opti-MEM. The plasmid/ LipofectAMINE mixture was then added to cells and incubated for 6 h, followed by replacement with unmodified cell culture medium. After 48 h, the cells were centrifuged at 100,000 g and supernatants were then collected. The resulting pellets were discarded. The resulting supernatants were then centrifuged at 100,000 g at 4 °C for 1 h. Supernatant protein concentrations were determined at a concentration of 500 μg/ml to initiate selection of resistant cells; culture medium was replaced at 2–3 day intervals. Approximately 3 weeks later, G418-resistant clones were isolated using an 8 × 8 mm cloning cylinder and analyzed individually for expression of GTPCH activity.

Preparation of Rabbit Antibodies to GTPCH Fusion Protein—The purified and concentrated GTPCH/maltose-binding protein fusion was used to develop polyclonal antibodies in each of two rabbits. Fusion protein in Freund’s complete adjuvant was injected intradermally into four dorsal sites (50 μg/injection) in each rabbit. After 28 days the animals were boosted by intradermal injection of an additional 100 μg of fusion protein in incomplete adjuvant, and thereafter, boosted by subcutaneous injection of 50 μg protein at 14-day intervals. Commencing at week 5, animals were bled every 2 weeks from a marginal ear vein. Serum was separated from the clotted blood and stored at −20 °C until use. Antibody titer was determined by enzyme-linked immunosorbent assay, and Western blot analysis established specificity for GTPCH activity.

RESULTS AND DISCUSSION

Phenylalanine and GFRP Modulate the Inhibition by DAHP of Immunostimulant-induced GTPCH Activity in RASMC—Feedback inhibition of the first enzyme in a given biosynthetic pathway by its end product is a common mechanism of biochemical regulation and conservation. Accordingly, it is well appreciated that BH4 regulates its own synthesis by serving as a feedback inhibitor of GTPCH (2). Feedback inhibition of GTPCH has been shown to require the participation of an auxillary protein, originally termed “p35” based on an apparent molecular mass of 35 kDa (6), but now known as GFRP, a pentameric protein composed of identical 9.5-kDa subunits (8). Because one important function of BH4 is as a rate-limiting cofactor for phenylalanine hydroxylase activity, it is significant that excess Phe can fully reverse the inhibition by BH4 of GTPCH/GFRP in vitro (6). In this regard, nature appears to have evolved a mechanism that ensures rapid detoxification of Phe. The observation that oral administration of Phe elicits an increase in plasma BH4 in normal individuals (20) implies that modulation of GTPCH activity by GFRP and Phe is operative in vivo. Specific reversal of GTPCH inhibition by Phe provides a simple in vitro diagnostic test of whether GFRP participates in the action of a given inhibitor of GTPCH.

RT-PCR revealed basal expression of GFRP mRNA in RASMC (Fig. 2). The PCR primers used were predicted to amplify 603 of the 631-bp full-length GFRP cDNA from rat liver (8); dideoxynucleotide sequencing confirmed 100% identity with the previously reported clone. Linearity of amplification during the 28 cycles of PCR used for Fig. 2 was verified by comparison of densitometry results when amplification proceeded for 25, 27, and 30 cycles (not shown). Omission of first strand cDNA synthesis prevented amplification of GFRP, indicating that mRNA was the source of the cDNA template for GFRP amplification. Amplification of a 213-bp fragment of GTPCH fusion protein by RT-PCR was used for monitoring RNA integrity as described previously (16). This 213-bp product is predicted to be specific for histone protein 3.3 cDNA versus genomic DNA, as the amplified genomic DNA would include an intron (16). Thus, the observation of a single 213-bp amplimer verifies a lack of relevant genomic DNA contamination in all samples tested.

As shown in Fig. 2A, specific expression of GFRP mRNA triphosphate was achieved by treatment with 30 μl of 1 N HCl and 10 μl of 0.1 M KI for 30 min at 37 °C in the dark. Samples were decolorized with 10 μl of freshly prepared 0.1 M ascorbic acid, vortexed, neutralized with NaOH (1 N, 30 μl), and dephosphorylated for 1 h at 37 °C with alkaline phosphatase (20 μl of 9 units/ml, dissolved in 50 mM Tris-HCl, 0.3 M KCl, 2.5 mM EDTA, 10% glycerol, pH 7.8) and neo-
increased upon treatment of RASMC with a combination of LPS (100 μg/ml) and rat IFN-γ (50 ng/ml). The increase in GFRP mRNA expression was detectable by 8 h, increased further by 24 h, and was abolished by inclusion of 0.3 μg/ml cycloheximide but not 3 μM dexamethasone. It is notable that induction of GFRP mRNA expression was elicited by IFN-γ alone, whereas LPS alone was ineffective. This pattern is opposite that for induction of GTPCH mRNA expression where LPS alone is effective, but IFN-γ is not (17). Densitometric analysis, normalized to histone 3.3 mRNA levels, revealed that 24 h treatment of RASMC with LPS/IFN triggered a significant (p < 0.01) increase in GFRP mRNA expression to 178 ± 5.3% that of basal levels (Fig. 2B).

Basal expression of GFRP mRNA in untreated RASMC is surprising because GTPCH protein is undetectable in these cells (see Ref. 10, Fig. 3, and below) and the only known function of GFRP is to regulate GTPCH activity. Whether basal GFRP mRNA translates into detectable levels of GFRP protein awaits future Western blot analyses, presently limited by the lack of an available GFRP-specific antibody. In any event, the observation that LPS/IFN induces GTPCH mRNA expression where LPS alone is effective, but IFN-γ is not (17). Disequilibrium analysis, normalized to histone 3.3 mRNA levels, revealed that 24 h treatment of RASMC with LPS/IFN triggered a significant (p < 0.01) increase in GFRP mRNA expression to 178 ± 5.3% that of basal levels (Fig. 2B).

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Stable GTPCH overexpression was accomplished by transfecting RASMC with a pcDNA3 expression plasmid containing an in-frame nucleotide insert that encodes rat GTPCH preceed by six His residues. Positive clones were selected in G418; clone G5 was exclusively used in this study. Basal expression of GTPCH protein was apparent from Western blot analysis of extracts from His6-GTPCH transfected cells (Fig. 3). GTPCH expression in transfected cells was revealed by a single strong band of 28–29 kDa, the predicted mass of GTPCH subunits, on Western blots performed using a polyclonal rabbit anti-GTPCH serum raised against purified bacterial-expressed GTPCH/MBP. In contrast, extracts of untransfected cells did not have detectable GTPCH protein. Nonetheless, treatment of untransfected cells with LPS/IFN for 24 h consistently elicited the appearance of a single 28–29-kDa protein band that exhibited a staining intensity 2–3-fold less than that observed basally from His6-GTPCH-transfected cells. LPS/IFN-treatment did result in a detectable further increase in GTPCH protein expression in His6-GTPCH transfected cells. DAHP alone or in combination with the dihydrofolate reductase inhibitor methotrexate (an inhibitor of the pterin salvage pathway, Ref. 2), did not influence the extent of GTPCH expression in LPS/IFN-treated control or overexpressing cells. The latter finding argues that the well known ability of DAHP to inhibit BH4 synthesis occurs independently of a reduction in GTPCH protein mass.

As shown in Fig. 4, the intracellular levels of total bipterin (BH4 and more oxidized species) was below detection limits in control untreated RASMC (<0.1 ng/10^5 cells). Despite abundant expression of GTPCH, BH4 was only detected in untreated His6-GTPCH-transfected cells when serum (data not shown) or LPS/IFN (Fig. 4) was added to the cell culture medium. This suggests that GTPCH expression alone is insufficient for BH4 synthesis in G5 cells and another LPS/IFN- or serum-induced factor is required. In any event, in Phe-free medium, LPS/IFN treatment of control RASMC elicited intracellular bipterin accumulation at a level ~25% of that observed in LPS/IFN-treated His6-GTPCH-transfected cells (Fig. 4). Notably, Phe (0.3 or 3.0 mM) caused a >100% increase in the bipterin content of LPS/IFN-treated control RASMC. Implicit in this finding is that intracellular BH4 reaches a concentration in LPS/IFN-treated control cells that is sufficient to elicit feedback inhibition when Phe is absent from the cell culture medium. Also implicit is that LPS/IFN-treated RASMC possess

2 S. S. Gross and L. Xie, unpublished observation.
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DAHP-mediated inhibition NO synthesis is consistent with a role for GFRP in DAHP action. Selectivity of Phe action is further implied by our finding that although 3 mM Phe reduced the inhibitory effect of DAHP on LPS/IFN-induced NO synthesis by an order of magnitude in control RASMC, Phe (0 versus 3 mM) did not alter the relative inhibitor potency of an arginine-based competitive blocker of NOS, l-NMA (data not shown).

Phenylalanine and GFRP Modulate the Inhibition by DAHP of Purified GTPCH—Recombinant GTPCH was expressed in E. coli as a 71-kDa fusion protein with mannose-binding protein (rGTPCH) and purified by affinity chromatography on amylose resin. Purified rGTPCH was found to be catalytically competent and produced neopterin triphosphate with a typical specific activity of 10–15 pmol/min/mg protein at 37 °C.

At a concentration of 1 mM, DAHP elicited a 50% inhibition of dihydronopterin triphosphate production by purified rGTPCH in a defined in vitro assay system (Fig. 7). In the presence of this inhibitory concentration of DAHP, rGTPCH activity was reduced by a further 45% upon addition of 150 μM of crude GFRP-containing cytosolic protein (prepared from LPS/IFN-treated RASMC). Whereas 3 mM Phe did not diminish the extent of rGTPCH inhibition caused by 1 mM DAHP alone, Phe completely reversed the further inhibition caused by addition of cytosolic protein. Thus, a component of RASMC cytosol, presumably GFRP, was found to enhance the inhibitory potency of DAHP by a Phe-reversible mechanism. The total biopterin concentration of RASMC cytosol included in the experiment was found to be 6.3 μM (the sum of biopterin, BH2 and BH4; mean value, n = 3). Assuming that all biopterin was present as BH4 and that all BH4 was bound to GFRP (which is certainly an overestimate), then GFRP-BH4 complexes could maximally occupy only 2.2% of total GTPCH (280 nM). Actually, GFRP-BH4 complexes should be well below this theoretical maximum because GFRP-dependent inhibition has only been detected when BH4 concentrations exceed 1 μM (8). Thus, the low abundance of BH4 in cytosol argues strongly against the possibility that pre-existing BH4-GFRP complexes were responsible for the Phe-reversible inhibition of GTPCH described by Fig. 7.

In agreement with findings presented in Fig. 7, DAHP was found to cause a dose-dependent inhibition of rGTPCH with an IC50 of ~1 μM (Fig. 8A). Addition of crude cytosol increased the inhibitory potency of DAHP, manifest by a leftward shift in the DAHP concentration-inhibition curve and a reduction in IC50 to 250 μM. To explore whether GFRP is the specific cytosolic principle that confers increased inhibition by DAHP, we sought to elicit the selective transfer of cytosolic GFRP to rGTPCH and then to assess whether this transfer results in a perpetuated increase in DAHP’s inhibitory potency. Toward this end, we incubated highly purified rGTPCH with crude cytosol from RASMC for 1 h at 4 °C in buffer containing 200 mM NaCl, a salt concentration that was earlier shown to disrupt GTPCH/GFRP interaction (8). This high salt buffer would predictably release GFRP from any pre-existing complexes in the RASMC cytosol, making GFRP available for subsequent interaction with rGTPCH. It was further hypothesized that a subsequent 10-fold reduction in salt concentration, by sample dilution, would allow for GFRP complex formation with GTPCH, yielding rGTPCH/GFRP complexes. Consistent with GFRP transfer to rGTPCH, subsequent repurification rGTPCH on amylose resin yielded an enzyme with greater sensitivity to DAHP than observed with naive rGTPCH, but equivalent DAHP sensitivity to that found of cytosolic protein. Thus, a component of RASMC cytosol, presumably GFRP, was found to enhance the inhibitory potency of DAHP by a Phe-reversible mechanism. The total biopterin concentration of RASMC cytosol included in the experiment was found to be 6.3 μM (the sum of biopterin, BH2 and BH4; mean value, n = 3). Assuming that all biopterin was present as BH4 and that all BH4 was bound to GFRP (which is certainly an overestimate), then GFRP-BH4 complexes could maximally occupy only 2.2% of total GTPCH (280 nM). Actually, GFRP-BH4 complexes should be well below this theoretical maximum because GFRP-dependent inhibition has only been detected when BH4 concentrations exceed 1 μM (8). Thus, the low abundance of BH4 in cytosol argues strongly against the possibility that pre-existing BH4-GFRP complexes were responsible for the Phe-reversible inhibition of GTPCH described by Fig. 7.
when rGTPCH activity was assayed in the continuous presence of RASMC cytosol (Fig. 8A).

Coomassie Blue-stained SDS-PAGE of rGTPCH, either naive (lane 1), or repurified on amylose resin following incubation with cytosol (lane 2), is depicted in Fig. 8B. It is notable that this SDS-PAGE used the very preparations whose activity is described by Fig. 8A. Naive rGTPCH was found to exclusively comprise a 71-kDa protein band corresponding to GTPCH/MBP fusion (labeled A) and a modest amount of contaminating 42-kDa free MBP (labeled B). In addition to these dominant proteins, two additional protein bands were observed following repurification from the cytosol incubate (lane 2); these correspond to 28-kDa-free GTPCH (commencing with N-terminal Gly6, labeled C) and a 9–10-kDa protein, the predicted size of GFRP (labeled D).

Fig. 8. GFRP-containing cytosol enhances the potency of DAHP for inhibiting activity of recombinant GTPCH (rGTPCH). Panel A, the inhibitory potency of DAHP was assessed on naive rGTPCH (■), rGTPCH in the presence of crude GFRP-containing cytosol from LPS/IFN-treated RASMC (150 μg total protein, ▲), and rGTPCH that was repurified after exposure to cytosol as described in “Experimental Procedures” (▲). Samples contained 2 μg of GTPCH. Points are means ± S.E. (n = 3). Panel B, SDS-PAGE showing the purification of both rGTPCH and repurification of rGTPCH after RASMC cytosol treatment. rGTPCH/MBP fusion protein was expressed in E. coli and purified to homogeneity by chromatography on amylase affinity resin (lane 1). Two bands were observed: a 71-kDa band corresponding to GTPCH/MBP fusion (labeled A) contaminated by a modest amount of 42-kDa-free MBP (labeled B). Two additional bands were observed following repurification from the cytosol incubate (lane 2); these correspond to 28-kDa-free GTPCH (commencing with N-terminal Gly6, labeled C) and a 9–10-kDa protein, the predicted size of GFRP (labeled D).

Fig. 9. Double-reciprocal plot indicates that DAHP is a relatively weak competitive inhibitor of purified GTPCH. Factor Xa was used to cleave rGTPCH/MBP fusion protein. Free GTPCH was purified from MBP as described in the text, and 0.40 μg was incubated for 30 min at 37 °C with the indicated concentrations of GTP (25–4000 μM), in the absence or presence of 1 or 2 mM DAHP. Points are means of triplicate determinations of dihydroneopterin triphosphate, measured using the fluorescence microtiter plate assay. Linear regression analysis of these data reveal that the Kₘ and Vₘₘₜₜ for GTP utilization are 192 μM and 11.90 nmol/min/mg protein, respectively. Inhibition by DAHP is shown to be purely competitive with GTP with a Kᵢ of 756 μM.

Fig. 10. Schematic to depict the proposed role of GFRP as mediator of GTPCH inhibition by DAHP. The model is speculative, based on our limited understanding of the mechanism by which BH4 mediates feedback inhibition of GTPCH. It is assumed that DAHP serves as a BH4-mimic in this system; see text for details.

in potency of inhibition by DAHP, argues strongly for GFRP as the mediator of DAHP’s potent inhibition of GTPCH.

Although GFRP appears to confer potent inhibition of GTPCH by DAHP, it is equally apparent that the weaker inhibition by DAHP is GFRP-independent. Indeed, purified naive rGTPCH from E. coli is inhibited by elevated concentrations of DAHP although it is clearly devoid of any protein having the molecular mass of GFRP. Moreover, it is unlikely that E. coli even possess GFRP, inasmuch as bacterial isoforms of GTPCH are not susceptible to feedback inhibition by BH4. As shown in Fig. 9, the lower potency inhibition by DAHP observed with purified rGTPCH results from a specific increase in Kᵢ for
GTP (from 192 μM in the absence of DAHP to 560 μM with 1 mM DAHP) without a detectable change in V_{max}. These findings reveal that in addition to the more potent GFRP-dependent inhibition of GTPCH, DAHP is a weak competitive inhibitor of GTPCH with K_{I} = 756 μM.

The present findings reveal that dual mechanisms mediate inhibition of GTPCH by DAHP. We show here for the first time that the more potent mode of GTPCH inhibition by DAHP, i.e. that which occurs at lowest DAHP concentration, requires GFRP and presumably arises from engagement of regulatory mechanisms that otherwise mediate feedback inhibition of GTPCH by BH4. This view is supported by multiple lines of evidence in RASMC. In summary, we show that: (1) GFRP mRNA is expressed basally and induced by immunological stimuli; (2) GFRP is functional, conferring feedback inhibition by BH4; (3) elimination of GFRP inhibitory function (either by addition of Phe or by overexpression of GTPCH) causes an 8-fold reduction in the potency of DAHP as an inhibitor of GTPCH; (4) pure GFRP-free rGTPCH is weakly inhibited by DAHP; (5) addition of GFRP-containing cytosol to pure rGTPCH enhances the inhibitory potency of DAHP (this effect of cytosol is abolished when Phe is added to functionally inactivate GFRP); and (6) chemical transfer of GFRP to pure GTPCH causes a sustained increase in the potency of DAHP. Taken together, these findings strongly implicate a role for GFRP in the inhibition of GTPCH by DAHP. Previously, GFRP was considered only as a participant in the mechanism of feedback inhibition by BH4 expression, and it was not known to be subject to regulation by immunostimulants.

The initial impetus for our considering a role for GFRP in the mechanism by which DAHP inhibits GTPCH, was a perceived structural homology between DAHP and BH4. Indeed, our present findings therefore support the view that DAHP acts as a mimic of BH4 by commandeering the erastwhile GFRP-mediated feedback inhibitory pathway. Consideration of the rudimentary knowledge on the mechanism by which GFRP and BH4 function together to elicit feedback inhibition of GTPCH provides insight and helps to focus questions regarding DAHP. A preliminary model for how DAHP may inhibit GTPCH is presented in Fig. 10.

The high resolution crystal structure of GTPCH from E. coli reveals a face-to-face dimer of toroidal homopentamers (5), as depicted in Fig. 10. Ten active sites are present around the periphery of the toroid; each is composed of three units, two adjacent subunits from one pentamer and an overlapping subunit from the more distal pentamer. Assuming a GTPCH-GFRP subunit stoichiometry of 1, and given that GFRP is a homopentamer (9), it is reasonable to speculate that one GFRP pentamer assembles on each face of GTPCH (only the GFRP pentamer that lies above the plane of GTPCH is visible in Fig. 10). Previous studies suggest that assembly of this complex is triggered by Phe or excess BH4 resulting in active or inactive GTPCH, respectively. Presumably, DAHP can substitute for BH4 to promote formation of the catalytically inactive complex. Whether the binding site for DAHP (and BH4) resides on GTPCH or GFRP in isolation or whether binding site formation requires assembly of the GTPCH-GFRP complex awaits clarification. The relationship between binding site occupancy and percent inhibition of GTPCH also remains to be determined. Does a progressive increase in the percent occupancy of sites by DAHP result in a corresponding percent increase in GTPCH inhibition? Alternatively, is it the case that occupancy of a single DAHP binding site is sufficient to inhibit activity at all ten active sites? It is notable that Phe restores catalytic activity to the BH4-inhibited GFRP-GTPCH complex, without disrupting the multimeric complex (6). Although we know that Phe reverts the inhibitory action of DAHP, it is assumed but unproven that the GTPCH-GFRP complex is retained. Also, it remains to be determined whether Phe restores activity by binding to a single site per GTPCH-GFRP complex (as shown for simplicity in Fig. 10), or more likely, by binding a single site per GFRP or GTPCH monomer. Future structural studies will be needed to reveal molecular details of the mechanism by which Phe triggers formation of the disintegrated complex.

These findings advance our understanding of the cellular mechanisms that regulate tetrahydrobiopterin synthesis, but raise new issues. It will be important to learn the extent to which cellular distribution of GFRP modulates the ability of a cell to produce BH4. Previously, GFRP was considered as a sensor for elevated levels of BH4 and Phe, we must now consider it as a pharmacological modulator in a broader sense. Moreover, the relative potency of DAHP to inhibit BH4 synthesis in a given tissue will dependably predict the position on the tissue’s GFRP content.

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