Purification and Cloning of the GTP Cyclohydrolase I Feedback Regulatory Protein, GFRP*

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The activity of GTP cyclohydrolase I, the initial enzyme of the de novo pathway for biosynthesis of tetrahydrobiopterin, the cofactor required for aromatic amino acid hydroxylations and nitric oxide synthesis, is sensitive to end-product feedback inhibition by tetrahydrobiopterin. This inhibition by tetrahydrobiopterin is mediated by the GTP cyclohydrolase I feedback regulatory protein GFRP, previously named p35 (Harada, T., Kagamiyama, H., and Hatakeyama, K. (1993) Science 260, 1507-1510), and L-phenylalanine specifically reverses the tetrahydrobiopterin-dependent inhibition. As a first step in the investigation of the physiological role of this unique mechanism of regulation, a convenient procedure has been developed to co-purify to homogeneity both GTP cyclohydrolase I and GFRP from rat liver. GTP cyclohydrolase I and GFRP exist in a complex which can be bound to a GTP-affinity column from which GTP cyclohydrolase I and GFRP are separately and selectively eluted. GFRP is dissociated from the GTP agarose-bound complex with 0.2 M NaCl, a concentration of salt which also effectively blocks the tetrahydrobiopterin-dependent inhibitory activity of GFRP. GTP cyclohydrolase I is then eluted from the GTP-agarose column with GTP. Both GFRP and GTP cyclohydrolase I were then purified separately to near homogeneity by sequential high performance anion exchange and gel filtration chromatography. GFRP was found to have a native molecular mass of 20 kDa and consist of a homodimer of 9.5-kDa subunits. Based on peptide sequences obtained from purified GFRP, oligonucleotides were synthesized and used to clone a cDNA from a rat liver cDNA library by polymerase chain reaction-based methods. The cDNA contained an open reading frame that encoded a novel protein of 84 amino acids (calculated molecular mass 9665 daltons). This protein when expressed and may play a role in regulating not only phenylalanine metabolism in the liver, but also the production of biogenic amine neurotransmitters as well as nitric oxide synthesis.

6R-L-erythro-5,6,7,8-Tetrahydrobiopterin (BH₄) is the required cofactor for the members of the family of aromatic amino acid hydroxylases (1) and also for the three different isoforms of nitric oxide synthase (2). The de novo pathway for biosynthesis has been well characterized (3), yet little is known of the regulation of this pathway in vivo and how this regulation impinges on the activities of the enzymes for which it is required. Studies of acute and chronic regulation of BH₄ levels in vivo have generally focused on the first enzyme of the BH₄ biosynthetic pathway, GTP cyclohydrolase I, since changes in its activity usually mediate or closely correlate with changes in BH₄ levels (3). Genetic deficiencies of GTP cyclohydrolase I activity have been described which result in hyperphenylalaninemia due to the lack of BH₄ (4). Recently, a hereditary form of progressive dystonia was shown to be caused by a mutation in the GTP cyclohydrolase I gene resulting in decreased dopamine synthesis in the central nervous system (5). In the adrenal medulla and cortex, catecholamine synthesis as well as BH₄ levels and GTP cyclohydrolase I activity have been shown to be hormonally regulated (6), probably through the action of cAMP-mediated pathways leading to increased protein synthesis (7). Recently, reserpine, which had previously been found to increase BH₄ levels and GTP cyclohydrolase I activity in the adrenal cortex (8), was shown to increase levels of GTP cyclohydrolase I mRNA in peripheral and central neurons (9). Activation of the immune system results in the production of interferon-γ which then stimulates an increase in GTP cyclohydrolase I activity in macrophages resulting in increased extracellular dihydronorleptin levels in humans and intracellular BH₄ levels and BH₄-dependent nitric oxide synthase in rodent (10). GTP cyclohydrolase I mRNA and BH₄ synthesis are also induced by interferon-γ in lymphocytes (11), by interleukin-1β in smooth muscle cells (12), and by cytokines in endothelial cells where it regulates the activity of the endothelial form of nitric oxide synthase (13). Administration of bacterial endotoxin (lipopolysaccharide) to rats has been shown to increase GTP cyclohydrolase I activity and BH₄ levels in cerebellum, liver, spleen, and adrenal gland (14).

The mechanism of two other types of acute regulation of BH₄ biosynthesis, phenylalanine-induced increases in BH₄ levels

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U53710.

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1 The abbreviations used are: BH₄, 6R-L-erythro-tetrahydrobiopterin; BSA, bovine serum albumin; DTT, dithiothreitol; GFRP, GTP cyclohydrolase I feedback regulatory protein; HPLC, high performance liquid chromatography; PCR, polymerase chain reaction; SSPE, saline-sodium phosphate-EDTA buffer; Tricine, N-tris(hydroxymethyl)methylglycine.
GTP cyclohydrolase I and its associated feedback regulatory concentrations (Fig. 1).

Defect in BH4 biosynthesis, suggesting that phenylalanine ingestion a phenylalanine challenge but not in children who had a defect in BH4 synthesis when it is present in higher than needed concentrations to increase in response to dietary phenylalanine by in vivo and in vitro experiments (18). Thus, this putative auxiliary protein, which is hydroxylated to tyrosine in a BH4-dependent reaction catalyzed by phenylalanine hydroxylase (PAH), specifically reverses the inhibitory effect of BH4, while having no direct effects in the absence of BH4.

A link between the phenylalanine stimulatory effect and the end-product feedback regulation by BH4, have recently been clarified. In 1975, Leeming et al. (15) discovered that plasma BH4 levels in normal children increased rapidly following a phenylalanine challenge but not in children who had a defect in BH4 biosynthesis, suggesting that phenylalanine might stimulate BH4 biosynthesis. This possibility was strengthened by the finding that administration of phenylalanine to rats, together with labeled quinose to label GTP pools, stimulated the incorporation of label into BH4 (16). However, in the ensuing years, the mechanism of the phenylalanine effect remained enigmatic since phenylalanine does not stimulate GTP cyclohydrolase I activity in vitro nor does it have any effects on the other enzymes of the de novo pathway.2 In what was considered to be an unrelated type of regulation, it has long been known that GTP cyclohydrolase I activity was negatively regulated by the end product of the pathway, BH4, and it was assumed that this was an intrinsic property of the enzyme (17). A link between the phenylalanine stimulatory effect and the BH4 inhibitory effect was established recently when Harada et al. (18) found that pure, recombinant GTP cyclohydrolase I was not inhibited by BH4 and that another protein, called p35 because of its apparent molecular mass, was present in rat liver extracts and conferred BH4-dependent inhibitory sensitivity to GTP cyclohydrolase I. Furthermore, it was also found that the BH4-dependent inhibitory activity could be reversed by l-phenylalanine (18). Thus, this putative auxiliary protein, when complexed to GTP cyclohydrolase I, constitutes a unique regulatory system allowing hepatic phenylalanine hydroxylation to increase in response to dietary phenylalanine by increasing synthesis of the rate-limiting cofactor and turning off BH4 synthesis when it is present in higher than needed concentrations (Fig. 1).

In this report, a convenient method is described to co-purify GTP cyclohydrolase I and its associated feedback regulatory protein to homogeneity which facilitated the molecular cloning of the regulatory protein. Our results suggest that this regulatory protein may play an important role not only in regulating phenylalanine metabolism, but also in the regulation of catecholamine and serotonin synthesis, as well as nitric oxide production. It is suggested that this protein should more properly be named GTP cyclohydrolase I feedback regulatory protein, or GFRP, rather than p35.

### EXPERIMENTAL PROCEDURES

#### Materials

GTP, GTP immobilized on 4% agarose by coupling periodate oxidized GTP to an adipic acid dihydrazide linker, amino acids, and dihydropterin triphosphate were obtained from Sigma. Intestinal alkaline phosphatase was from Calbiochem. Pterins were all obtained from B. Schircks Lab (Jona, Switzerland).

#### Enzyme Assays

**GTP Cyclohydrolase I Activity**—Two different methods were used to determine GTP cyclohydrolase I activity which gave identical results for purified GTP cyclohydrolase I preparations. For crude enzyme preparations and in some experiments with purified enzyme, activity was determined by measurement of neopterin produced after oxidation and dephosphorylation of the enzymatic product, dihydropterin phosphatase in the presence of contaminating phosphatases. In brief, assays were carried out exactly as described above except that reactions were terminated by addition of 5 μl of 1 M H3PO4 or 1 M NaOH. The samples were then mixed, centrifuged briefly, and dephosphorylated by addition of 1 μl of intestinal alkaline phosphatase (10 units) and incubation for 20 min at 37°C. Finally, 100 μl of 1 M H3PO4 were added, and samples were clarified by centrifuging at 10,000 × g for 2 min in a microcentrifuge. Neopterin was measured by reverse phase HPLC with fluorometric detection as described previously (20).

For more highly purified preparations, GTP cyclohydrolase I activity was determined by measurement of neopterin triphosphate. This method could not be used for less purified samples due to the lower sensitivity of fluorescence detection in the required HPLC solvent systems and to the presence of contaminating phosphatases. In brief, assays were carried out exactly as described above except that reactions were terminated by addition of 100 μl of 1 M H3PO4 and dihydropterin triphosphate was oxidized to neopterin triphosphate by adding 10 mg of MnO2 and incubating for 10 min at room temperature. After centrifugation at 10,000 × g for 2 min, supernatants were stored on ice until analyzed. Preliminary experiments indicated that samples could be kept on ice for up to 2 h without significant dephosphorylation occurring. Neopterin triphosphate was measured by anion exchange HPLC (Whatman SCX, 0.4 M potassium monophosphate, 0.8 M KCl at 2°C) with fluorometric detection as described by Zagalak et al. (21).

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**Materials**

Neopterin triphosphate was oxidized to neopterin as described above, it was necessary to wash the reverse phase HPLC column for an additional 10 min to elute biotin prior to injection of the next sample. Addition of biotin (or other pterins) during the alkaline phosphatase step had no significant effects on the yield of product. Assays were routinely carried out with two different concentrations of the material being assayed to ensure that the inhibitory response was proportional to the amount of GFRP added.

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2 S. Milstien, unpublished observations.
Co-purification of GTP Cydohydrolase I and GFRP

All procedures were carried out at room temperature unless indicated otherwise.

Extract—Frozen rat livers from male Sprague-Dawley rats (300 g, Harlan Bioproducts for Science) were suspended in 900 ml of extraction buffer B (50 mM KPO4, pH 7.4, 1 mM DTT, 1 mM EDTA, 2 μg/mL each aprotinin, leupeptin, pepstatin, 0.2 μM phenylmethylsulfonyl fluoride, 0.1% Tween 20; 10% glycerol) in a beaker placed in an ice bath. After 30 min, livers were minced with a scissors and homogenized in a Waring glass Dounce homogenizer. The extract was heated to 65°C in a boiling water bath in 200-mL portions while stirring with a thermometer. The heat-treated supernatant was placed in a beaker in an ice bath with a stirring bar and a pH electrode. The pH was adjusted to 5.0 by dropwise addition of glacial acetic acid. After stirring beaker in an ice bath with a stirring bar and a pH electrode. The pH was adjusted to 5.0 by dropwise addition of glacial acetic acid. After stirring, the supernatant was discarded. The dark brown pellet was dissolved in small portions over 10 min. After stirring for an additional 20 min, the sample was centrifuged for 15 min at 20,000 × g, and the supernatant from both centrifugations was combined.

Acid Precipitation—The heat-treated supernatant was placed in a beaker in an ice bath with a stirring bar and a pH electrode. The pH was adjusted to 5.0 by dropwise addition of glacial acetic acid. After stirring for an additional 30 min, the sample was centrifuged for 30 min at 20,000 × g, and the supernatant was discarded. The dark brown pellet was rapidly resuspended in 50 ml of cold buffer B by homogenizing in a glass Dounce homogenizer.

Ammonium Sulfate Precipitation—While stirring in an ice bath, finely powdered ammonium sulfate (0.134 g/mL) was added to the redissolved acid precipitate in small portions over 10 min. After stirring for an additional 20 min, the sample was centrifuged at 20,000 × g for 20 min, and the pellet was discarded. An additional portion of ammonium sulfate (0.179 g/mL) was then added to the supernatant as above to precipitate GTP cydohydrolase I and the associated GFRP activity. After centrifugation for 20 min at 20,000 × g, the pellet was dissolved in 10 ml of buffer B.

Dialysis—The resuspended ammonium sulfate pellet was dialyzed overnight at 4°C against 1 liter of buffer B (Spectra/por, 6–8000 MWCO). Dialyzed samples could be rapidly frozen and stored at −70°C for several weeks without loss of activity.

Affinity Chromatography—In preliminary experiments, it was found that in contrast to previous claims that eukaryotic GTP cydohydrolase I could not be purified by affinity chromatography on a GTP-affinity matrix, rat liver GTP cydohydrolase I activity purified 200-fold through the dialysis step described above was found to strongly bind to GTP-agarose affinity media and could be eluted specifically with GTP. However, when this was done, almost all of the BH4-dependent inhibitory activity that was present in the dialyzed sample and bound to the GTP-agarose column was eluted by GTP together with GTP cydohydrolase I activity (data not shown). Further attempts to separate the inhibitory activity from the GTP cydohydrolase I activity by anion exchange chromatography or by gel filtration were not successful. However, it was discovered that it was possible to dissociate GFRP from the GTP cydohydrolase I-GFRP complex by washing the affinity column with 0.2 M NaCl prior to specific elution of GTP cydohydrolase I with GTP (see “Results”). In this protocol, the dialyzed sample was applied to a column of GTP agarose (1 ml) pre-equilibrated in buffer B. After washing with 50 ml of buffer B, GFRP was eluted with 10 ml of buffer containing 0.2 M NaCl. After a further wash with 1 ml of buffer B alone, the GTP cydohydrolase activity I was eluted with 10 ml of buffer B containing 5 mM GTP. The salt eluate and the GTP eluate were separately concentrated to small volumes in centrifugal concentrators (Centricron 10, Amicon). The GTP-affinity column was regeneranted by washing with 50 ml of water, 1 ml of 10% sodium dodecyl sulfate, 50 ml of water, and finally with 5 ml of 50% glycerol before storage at −20°C. It should be noted that the same column was reused more than 10 times without noticeable loss of GTP cydohydrolase I binding capacity.

Further Purification of GTP Cydohydrolase I—Affinity-purified GTP cydohydrolase I was purified to homogeneity in high yield by a two-step procedure using high performance anion exchange chromatography followed by high performance gel filtration. The concentrated, affinity-purified GTP cydohydrolase I (~1 ml) was diluted with 4 ml of buffer B and injected onto a Mono Q column (Pharmacia, HR 5/5) equilibrated with buffer B at a flow rate of 1 ml/min. The chromatography system consisted of an all-Titanium Gilson HPLC system with a Rainin Macintosh controller. The absorbance was monitored at 280 nm with a Gilson 121 UV detector. All runs were made at room temperature. After washing with buffer B until the absorbance reached base line, a linear gradient to 0.4 M NaCl in buffer B was run over 80 min, and 1-min fractions were collected. Groups of 4–5 fractions were collected and transferred to an ice bath until assayed. Fractions with the highest activity were pooled, and concentrated to 200 μl with Centricron 30 concentrators (Amicon). The concentrated GTP cydohydrolase I fractions were then purified by gel filtration on a Superose 6 (Pharmacia) column using the HPLC apparatus described above and eluted with buffer C (same as B, without protease inhibitors) containing 0.1 M NaCl at a flow rate of 0.3 ml/min. Fractions (0.3 ml) with the greatest activity were collected, concentrated as above, and stored in aliquots at −70°C.

Further Purification of GFRP—GFRP eluted from the GTP affinity column by 0.2 M NaCl was purified to homogeneity by a similar two-step HPLC procedure carried out in the reverse order, gel filtration followed by Mono Q chromatography. The NaCl eluate from the GTP-agarose column was concentrated to 200 μl with Centricron 10 concentrators and injected onto the HFLC gel filtration columns at a flow rate of 0.4 ml/min with buffer C (containing 0.1 M NaCl). A Zorbax GF-250 precolumn and column (DuPont NEN) were coupled in series with a Superose 6 column to increase the separation capacity. Fractions from the tandem gel filtration columns with the highest BH4-dependent GTP cydohydrolase I inhibiting activity were pooled, concentrated with Centricron 10 concentrators, and buffer-exchanged into buffer C with a PD-10 column (Pharmacia). Most of the GFRP activity was removed as described above for GTP cydohydrolase I. Fractions with GFRP activity were concentrated with a Centricron 10 concentrator and stored at −70°C.

Cloning and Expression of Rat Liver GFRP cDNA

Degenerate primers Sm3 (coding strand, ATGACGNNGTNAC-NCARCT) and Sm5 (noncoding strand, GYTGNNCCACNCNGTT-CAT) were used on DNA prepared from a rat liver cDNA library (provided by M. Brownstein) in the pCD SP877 vector. PCR amplification (96°C, 1 min; 55°C, 1 min; 72°C, 4 min; 30 cycles) gave a 0.19-kilobase product whose predicted amino acid sequence exactly matched the target peptide sequence. To amplify the 2′-end library pair of nested vector primers, up3 (AAGCTTCTCTAGATGTTGTCG) and up2 (TACGCGTCTACGGAGATGTTCG), were used with Sm5 and a nested primer based on the cDNA sequence, Sm7 (AGACCCCGTAC-TCAGCTTGG). To amplify the 3′-end of the cDNA, primers 1 and Sm6 (GACTGATGTTGATGTTTAA) were used in conjunction with vector primer dn2 (AATCCGTGCTAGTGTGTGGCACG). PCR products (96°C, 1 min; 60°C, 1 min; 72°C, 4 min; 30 cycles) of 250-1300 bases resulting from primers 1 and dn2 or up3 and Sm5 were size-selected on a 2% NuSieve gel and reamplified with primers dn2 and Sm6 or up2 and Sm7, respectively. The resulting products of about 650 bases (3′ end) and 230–330 bases (5′ end) were subcloned into pUC18 using a BamHI site in the cDNA sequence and either XhoI or PstI sites in the pUC-derived portion of the product and sequenced. To obtain a fragment containing the complete coding sequence, the PCR reactions were repeated using primers derived from the 5′ and 3′ ends of the cDNA and containing a build-in KpnI or XbaI site (GF3R, CCGTGACCCATGC-CCTACCTGTCACTGACCTCA; GFR4, GCCCTAGATGGCTTG-

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Other Assays

Protein was determined by the Coomassie dye binding assay (Pierce) with BSA as a standard.

Peptide Analysis

An aliquot of purified GFRP (8 μg) was dried in vacuo, redissolved in 50 μL of 8 M urea in 0.4 M ammonium bicarbonate, and then subjected to reduction, alkylation, and proteolytic digestion with 1.5 μg of modified trypsin (Promega) (22). The peptides in the resulting digest were fractionated by reverse-phase HPLC on a Vydac 218TP525 column (Separations Group, 2.1 × 250 mm) at 35°C using the gradient described by Fernandez et al. (23) on a System Gold HPLC equipped with a Model 507 autosampler, Model 126 programmable solvent module, and Model 168 diode array detector (Beckman). Solvent A was 0.1% trifluoroacetic acid in water, and solvent B was 0.1% trifluoroacetic acid in acetonitrile. Column effluent was monitored at 215 and 280 nm, and fractions were collected at 30-s intervals and stored at −70°C. Fractions (125 μL) containing tryptic peptides were applied in 30-μl aliquots to a Biorex (Applied Biosystems Inc.) treated glass fiber filter and dried prior to amino acid sequencing on a Model 477A pulsed-liquid protein sequencer equipped with a Model 120A PTH analyzer (ABI) using methods and cycles supplied by the manufacturer. Data were collected and analyzed on a Model 610A data analysis system (Applied Biosystems Inc.).
Purification and Cloning of GFRP

GATTAGCTGTGGTAGG). Digestion of the resulting product with KpnI and XbaI allowed it to be inserted into the KpnI and XbaI sites of the pTrxFus expression vector (Invitrogen) with the initiating methionine in-frame with the thioredoxin gene. Cleavage of the fusion protein with enterokinase should produce GFRP protein with two additional amino acids at the amino-terminal, valine and proline. Proper insertion of the fragment and the absence of PCR-generated mutations was confirmed by sequencing the expression vector construct. A GenBank search did not reveal any known proteins with significant homology to GFRP.

The GFRP-thioredoxin fusion protein was expressed in Escherichia coli. GI724 cells (Invitrogen) after transformation of the cells with plasmid expressing pTrxFus. The expression vector (Invitrogen) with the initiating methionine XbaI site of the pTrxFus expression vector (Invitrogen) with the initiating methionine XbaI allowed it to be inserted into the KpnI and XbaI sites of the pTrxFus expression vector (Invitrogen) with the initiating methionine in-frame with the thioredoxin gene. Cleavage of the fusion protein with enterokinase should produce GFRP protein with two additional amino acids at the amino-terminal, valine and proline. Proper insertion of the fragment and the absence of PCR-generated mutations was confirmed by sequencing the expression vector construct. A GenBank search did not reveal any known proteins with significant homology to GFRP.

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fractions corresponding to much lower molecular mass (data not shown). This observation raised the possibilities that the higher ionic strength buffer used in the gel filtration step might be causing dissociation of bound GFRP from GTP cyclohydrolase I and also that the actual molecular mass of GFRP might be less than the expected 35 kDa. The first possibility was investigated by examining the effect of increasing salt concentration on the BH4-dependent inhibition of affinity-purified GTP cyclohydrolase I-GFRP complex. As shown in Fig. 4, high salt concentration alone strongly inhibited GTP cyclohydrolase I activity, an effect which has previously been noted with human GTP cyclohydrolase I and was ascribed to a decrease in the affinity of the enzyme for GTP (27). However, intermediate salt concentrations, from 100–300 mM, had little or no direct effect on GTP cyclohydrolase I activity. C, SDS-polyacrylamide gel electrophoresis. Samples were separated on a 12% Tris-glycine gel (Novex), fixed, and then stained with Coomassie Blue. Lane 1, extract (40 μg); lane 2, heated supernatant (5 μg); lane 3, acid precipitate (26 μg); lane 4, dialyzed (26 μg); lane 5, GTP eluate (2 μg); lane 6, Mono Q pool (2 μg); lane 7, Superose 6 pool (1 μg).

The specificity of the pterin-dependent inhibition of GTP cyclohydrolase I by GFRP was examined using the purified
enzyme and regulatory protein. The natural 6R-BH4 isomer had the most potent inhibitory effect and was severalfold more potent than 7BH4. The concentration of 6R-BH4, which gave 50% inhibition of GTP cyclohydrolase I, was about 3 μM which is comparable to the half-maximally effective concentration of 2 μM previously found (18). In contrast to previous studies (18), however, all of the dihydropterins examined, including 7,8-dihydrobiopterin, 7,8-dihydronopterin, and sepiapterin, were nearly as potent as 6R-BH4 when assayed with purified GFRP (Table II). Furthermore, xanthopterin, which exists in solution as a hydrated dihydropterin, was also found to have potent inhibitory power. In contrast, other fully oxidized pterins that were tested, with the exception of pterin-6-carboxylic acid, had no GFRP inhibitory activity.

In the feedback inhibitory scheme for the action of GFRP in the regulation of GTP cyclohydrolase I activity (Fig. 1), L-phenylalanine abrogates the BH4-dependent inhibitory effect. As shown in Fig. 5, L-phenylalanine specifically and dose-dependently reversed the BH4-dependent inhibition of GTP cyclohydrolase I activity in the presence of purified GFRP. 0.4 mM L-phenylalanine restored nearly one-half of the GTP cyclohydrolase I activity inhibited by 10 μM BH4 and GFRP. Other amino acids, including D-phenylalanine, L-tyrosine, and L-tryptophan were ineffective, in agreement with previous studies using a crude GFRP preparation (18).

Cloning and Expression of GFRP—Purified GFRP was digested with trypsin, and the resulting peptides were isolated by reverse-phase HPLC. Three relatively long peptides were sequenced by automated Edman degradation, the sequences of which are indicated in Fig. 6. Oligonucleotide primers based on the peptide sequences were used for PCR screening of a rat liver cDNA library. After several rounds of PCR screening, a full-length cDNA was obtained which contained an open reading frame coding for an 84-amino-acid protein with a calculated molecular mass of 9665 daltons (Fig. 6). The deduced peptide sequence contained the complete sequences of the three isolated peptides. The GFRP cDNA was ligated into the pTrxFus vector and expressed as a 23-kDa thioredoxin fusion protein. The fusion protein was purified by affinity chromatography on phenylarsine oxide-modified agarose which specifically binds the vicinal diithiols of thioredoxin. The fusion protein demonstrated BH4-dependent GFRP activity which was specifically reversed or blocked by L-phenylalanine (Fig. 7). Pure, recombinant thioredoxin (Sigma) had no effect on either GTP cyclohydrolase I or GFRP activities. Enterokinase treatment cleaved the fusion protein into its constitutive components. However, GFRP activity of the fusion protein was lost after treatment with enterokinase although GFRP isolated from rat liver was not digested by enterokinase nor did it lose activity when treated in the same manner as the fusion protein. Cleavage of the fusion protein would yield recombinant GFRP with two additional amino acids at the amino-terminal which may interfere with proper folding or association with GTP cyclohydrolase I.
GTP cyclohydrolase I specifically eluted from the GTP-affinity column with GTP and then purified by anion exchange chromatography. The native molecular mass of GFRP was found to be 20 kDa by gel filtration, consisting of a homodimer of 9.5-kDa subunits as determined by SDS-polyacrylamide gel electrophoresis. It is likely that the higher molecular mass of 35 kDa previously found by gel filtration of crude rat liver extracts (18) was caused by slow dissociation of BH4 from the GTP cyclohydrolase I-GFRP complex.

Northern Blot Analysis—Because it appears as though there is a stoichiometric complex between GTP cyclohydrolase I and GFRP in the liver, it was important to determine whether GFRP is also associated with GTP cyclohydrolase I in other tissues. To this end, Northern blot analysis of GFRP mRNA levels was performed on several rat tissues. The GFRP probe detected a major mRNA species of 800 base pairs (Fig. 8). The minor bands at 1.4 and 6 kilobases presumably represent incompletely spliced RNAs. Testis, heart, brain, and lung had lower levels of GFRP mRNA, while levels in spleen and muscle were much lower.

**DISCUSSION**

GTP cyclohydrolase I activity is regulated in a unique feedback regulatory loop by tetrahydrobiopterin in conjunction with a regulatory protein. Our observation that GTP cyclohydrolase I and the small feedback regulatory protein GFRP exist in a tight complex has facilitated the purification of GFRP to homogeneity from rat liver. In the purification procedure described in this report, 200-fold purified GTP cyclohydrolase I-GFRP complex was bound to a GTP-affinity column from which the GFRP could be eluted selectively. GFRP was then purified to homogeneity in high yield, with a final purification factor of nearly 70,000-fold from rat liver. This was approximately the same extent of purification as was found for GTP cyclohydrolase I specifically eluted from the GTP-affinity column with GTP and then purified by anion exchange chromatography and gel filtration. The native molecular mass of GFRP was found to be 20 kDa by gel filtration, consisting of a homodimer of 9.5-kDa subunits as determined by SDS-polyacrylamide gel electrophoresis. It is likely that the higher molecular mass of 35 kDa previously found by gel filtration of crude rat liver extracts (18) was caused by slow dissociation of BH4 from the GTP cyclohydrolase I-GFRP complex.

**FIG. 6.** Nucleotide sequence of rat liver GFRP and deduced amino acid sequence. Amino acid sequences determined by peptide analysis are underlined.

**FIG. 7.** Tetrahydrobiopterin-dependent activity of recombinant GFRP-thioredoxin fusion protein. The activity of purified GTP cyclohydrolase I (0.2 µg) was determined in the absence or presence of 6R-BH4 (10 µM), purified GTP-thioredoxin fusion protein (12.4 µg), and either L-phenylalanine (5 mM) or o-phenylalanine (5 mM). Neither BH4 nor the amino acids, either alone or in combination, had any effect on GTP cyclohydrolase I activity in the absence of GFRP fusion protein (data not shown). The results shown are means ± S.E. of triplicate determinations.

**FIG. 8.** Detection of GFRP mRNA in various rat tissues. A nylon membrane containing polyadenylated RNA isolated from various rat tissues (2 µg/lane) was hybridized with a [32P]-labeled, PCR-generated DNA fragment of GFRP. Photo is a 5-h exposure on a Fuji phosphorimager. The sizes of the major bands are indicated by arrows.
GTP cyclohydrolase I activity by GFRP and BH₄. Fully active GTP cyclohydrolase I is indicated by the large circle. The binding of BH₄ to the GTP cyclohydrolase I-GFRP complex induces a conformational change which decreases activity. Phenylalanine displaces the bound BH₄ and restores activity. In the presence of high salt, GFRP dissociates from GTP cyclohydrolase I.

Fig. 9. Proposed mechanism for the regulation of GTP cyclohydrolase I activity by GFRP and BH₄. GTP cyclohydrolase I activity results from a decrease in catalytic activity rather than an alteration in affinity for GTP. The mechanism whereby association of GFRP with GTP cyclohydrolase I confers sensitivity to feedback inhibition by BH₄ has not yet been elucidated. A plausible scheme is presented in Fig. 9. GFRP and GTP cyclohydrolase I are considered to be normally tightly associated because only a small fraction of GFRP in liver extracts appears to be unbound, and mixing equimolar amounts of GFRP and GTP cyclohydrolase I is sufficient to generate a complex which is BH₄-sensitive. There is little evidence for binding of BH₄ to the GTP cyclohydrolase I-GFRP complex. However, when crude rat liver extracts were fractionated by gel filtration, fractions that contained BH₄-sensitive GTP cyclohydrolase I activity also contained BH₄ and there was no BH₄ associated with affinity-purified GFRP. The GTP cyclohydrolase I in the tripartite complex likely undergoes a conformational change which reduces the enzyme activity. It is proposed that l-phenylalanine specifically reverses the inhibitory activity by displacing BH₄ from the complex. L-Phenylalanine is shown bound to the GFRP-GTP cyclohydrolase I complex because, as discussed above, it does not cause release of GFRP from the complex when it is bound to GTP-agarose, and, in addition, it has been shown that l-phenylalanine alters GTP cyclohydrolase I activity, converting the kinetics with GTP from sigmoidal to hyperbolic (18). Finally, raising the ionic strength causes dissociation of the inhibitory complex and removes sensitivity to BH₄ as well as restoring full GTP cyclohydrolase I activity. The number of molecules of GFRP bound to decameric GTP cyclohydrolase I has not yet been established. Further characterization of the GFRP regulatory mechanism by physical and kinetic methods should be facilitated by the cloning and overexpression of larger amounts of GFRP. Studies are underway to determine the physiological role of GFRP in the regulation of other BH₄-dependent processes, such as biogenic amine neurotransmitter synthesis and nitric oxide production.

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Purification and Cloning of GFRP