Decameric GTP Cyclohydrolase I Forms Complexes with Two Pentameric GTP Cyclohydrolase I Feedback Regulatory Proteins in the Presence of Phenylalanine or of a Combination of Tetrahydrobiopterin and GTP*

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The activity of GTP cyclohydrolase I is inhibited by (6R)-1-erythro-5,6,7,8-tetrahydrobiopterin (BH₄) and stimulated by phenylalanine through complex formation with GTP cyclohydrolase I feedback regulatory protein (GFRP). Gel filtration experiments as well as enzyme activity measurements showed that the number of subunits of GFRP in both the inhibitory and stimulatory complexes is equal to that of GTP cyclohydrolase I. Because GFRP is a pentamer and GTP cyclohydrolase I was shown here by cross-linking experiments to be a decamer, the results indicate that two molecules of a pentameric GFRP associate with one molecule of GTP cyclohydrolase I. Gel filtration analysis suggested that the complex has a radius of gyration similar to that of the enzyme itself. These observations support our model that one molecule of GFRP binds to each of the two outer faces of the torus-shaped GTP cyclohydrolase I. For formation of the inhibitory protein complex, both BH₄ and GTP were required; the median effective concentration of BH₄ and GTP were 2 and 26 μM, respectively. BH₄ was the most potent of biopterins with different oxidative states. Among GTP analogues, dGTP as well as guanosine 5′-O-(3′-thiotriphosphate) exhibited similar inducibility compared with GTP, whereas other nucleotide triphosphates had no effect. On the other hand, phenylalanine alone was enough for formation of the stimulatory protein complex, and positive cooperativity was found for the phenylalanine-induced protein complex formation. Phenylalanine was the most potent of the aromatic amino acids.

GFRP is a regulatory protein for GTP cyclohydrolase I (EC 3.5.4.16), the first and rate-limiting enzyme of the biosynthetic pathway of BH₄ (1, 2). BH₄ is synthesized from GTP by three enzymes (2). BH₄ is an essential cofactor for tyrosine hydroxylase, a key enzyme for the biosynthesis of catecholamines; tryptophan hydroxylase, the first enzyme of the biosynthesis of serotonin; phenylalanine hydroxylase, the main enzyme of phenylalanine metabolism; and nitric-oxide synthase (2, 3). The biosynthesis of BH₄ is thought to be tightly regulated with regard to the activities of those enzymes because the intracellular levels of BH₄ are at or below the saturating levels for those enzymes (1). Despite the potential importance of the regulation, the molecular mechanisms are still unknown. A major advance in our understanding of this issue has been made by our identification of the regulatory protein GFRP (1). The discovery of GFRP and its effects on GTP cyclohydrolase I revealed how the biosynthesis of BH₄ is linked to the activity of phenylalanine hydroxylase (1). In the presence of GFRP, BH₄ inhibits the enzyme activity of GTP cyclohydrolase I, and the inhibition is reversed by phenylalanine. Elevated levels of phenylalanine thus stimulate the biosynthesis of BH₄ to fill the cofactor requirement of phenylalanine hydroxylase, the activity of which is known to be also stimulated by phenylalanine (4). These mechanisms provide the explanation at a molecular level of several in vivo phenomena about the close relationship between the levels of BH₄ and phenylalanine that were observed in normal individuals and patients with phenylketonuria (1, 5–7).

Rat GTP cyclohydrolase I was purified, and the kinetic analysis of the enzyme displayed positive cooperativity against GTP (8). This enzyme is a 300-kDa multimer composed of identical subunits with a subunit molecular mass of 25 kDa (8, 9), but its exact number of subunits has not yet been determined. Rat GFRP has been recently purified and cloned (10, 11). GFRP is a pentamer of identical subunits with a subunit molecular mass of 9.5 kDa (11).

The regulation of GTP cyclohydrolase I by GFRP occurs through ligand-induced complex formation between these two proteins, which is reversible (1). We showed by using an activity assay that GFRP is associated with GTP cyclohydrolase I both in the presence of BH₄ and GTP and in the presence of phenylalanine, whereas no protein complex is formed in the absence of these ligand molecules. In particular, both GTP and BH₄ are required for formation of the inhibitory protein complex, whereas phenylalanine alone is sufficient for formation of the stimulatory protein complex (1). Interestingly, phenylalanine reduces the positive cooperativity of GTP cyclohydrolase I in the presence of GFRP (1). However, detailed information regarding complex formation is lacking. We accordingly established a gel filtration method for analyzing complex formation. In this paper, we characterize the stoichiometry of both types of complexes and the ligand specificity for complex formation.

EXPERIMENTAL PROCEDURES

Materials—GTP was obtained from Yamasa (Chiba, Japan) and U. S. Biochemical Corp., and GDP and GTP*S were from Boehringer Mannheim. BH₄ was a generous gift from the Suntory Institute for Medicinal Research and Development (Gunma, Japan). BH₄ was purchased from Schircks Laboratories (Jona, Switzerland). t-Biopterin and dGTP were from Sigma. Recombinant rat GTP cyclohydrolase I and GFRP were prepared as described previously (1, 11).
Complex Formation between GFRP and GTP Cyclohydrolase I

Determination of Extinction Coefficients of GTP Cyclohydrolase I and GFRP—Absorbance spectra of GTP cyclohydrolase I and GFRP were measured with a Perkin-Elmer Lambda 2 spectrophotometer. The extinction coefficients of GTP cyclohydrolase I and GFRP were determined to be $E_{m,280} = 0.72$ and $1.23 \text{ (mg/ml)}^{-1} \text{ cm}^{-1}$, respectively, based on the mass data obtained by amino acid analysis. Taking the subunit molecular weights of GTP cyclohydrolase I and GFRP as 25,784 and 9946 from the corresponding DNA sequences (1, 9, 11), we calculated the molar extinction coefficients to be $E_{m,280} = 1.86 \times 10^4$ and $1.22 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. All the molar concentrations of GFRP and GTP cyclohydrolase I used in this paper are expressed as those of their subunits.

Cross-linking of GTP Cyclohydrolase I—Cross-linking reactions were performed in 500 $\mu$1 of 200 mM triethanolamine HCl buffer (pH 8.5) containing 0.1 mM dithiothreitol at a GTP cyclohydrolase I concentration of 0.1 mg/ml. The reaction mixture was preheated on ice. To the mixture was added freshly prepared dimethyl suberimidate to concentrations varying from 0.05 to 1.0 mg/ml. The mixture was incubated at room temperature for 3 h. Protein was precipitated using a deoxycholate/Cl$_2$COCOOH procedure (12). The precipitates were washed with ethyl ether to remove Cl$_2$COCOOH and dissolved in electrophoresis sample buffer. Cross-linked protein was resolved by electrophoresis on 5% (w/v) polyacrylamide gels in the presence of SDS (13). After electrophoresis, protein was stained with Coomassie Brilliant Blue R-250 (Bio-Rad).

Assay of GTP Cyclohydrolase I Activity—The activity of GTP cyclohydrolase I was assayed as described (8, 14). The reaction mixture (50 $\mu$1) contained 50 mM Hepes-KOH buffer (pH 7.2), 0.2 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM bovine serum albumin, 200 $\mu$M GTP, and recombinant GTP cyclohydrolase I. The reaction was carried out at 25 °C for 30 min.

Gel Filtration—Assay for the complex formation between GFRP and GTP cyclohydrolase I was performed using gel filtration, which enabled us to separate the complex formed from free GFRP. Protein samples were separated on a 1 × 30-cm column of Superdex 75 (Amersham Pharmacia Biotech) or a 7.8 mm × 30-cm column of TSK G3000SW$_{XL}$ (Tosoh, Tokyo, Japan). The columns were equilibrated with 50 mM Hepes-KOH buffer (pH 7.2) containing 0.2 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and ligands indicated in the figure legends. Elution was performed at a flow rate of 0.8 ml/min for Superdex 75 and 1.0 ml/min for G3000SW$_{XL}$ at room temperature. The eluate was monitored at 280 nm. The peak heights of each GFRP and GTP cyclohydrolase I were linear at least in the range of protein injected between 0.1 and 0.8 nmol when the proteins were separately filtered through the gel matrices in the presence of phenylalanine or a combination of BH$_4$ and GTP as well as in the absence of any ligands.

RESULTS AND DISCUSSION

Subunit Number of Rat GTP Cyclohydrolase I

*Escherichia coli* GTP cyclohydrolase I was shown to be a decamer by crystallographic studies (17). Considering the high sequence similarity between *E. coli* (18) and rat (9) GTP cyclohydrolase I and their similar native molecular weights (8, 17), we hypothesized that rat GTP cyclohydrolase I also was a decamer. To determine the subunit number of GTP cyclohydrolase I, we performed cross-linking experiments using dimethyl suberimidate, a homobifunctional imidoester that is able to cross-link lysine residues. The cross-linking reagent was used at a variety of concentrations to obtain proteins cross-linked to variable extents. The cross-linked proteins were then analyzed by SDS-polyacrylamide gel electrophoresis under the conditions established by Weber and Osborn (13). As shown in Fig. 1, significant intersubunit cross-linking of GTP cyclohydrolase I was obtained at a dimethyl suberimidate concentration of 0.05 mg/ml. As higher concentrations of dimethyl suberimidate were used, the protein was further cross-linked. At a dimethyl suberimidate concentration of 1.0 mg/ml, the proteins were almost completely cross-linked and converged to one species with a molecular mass of 280 kDa. When the estimated molecular masses of the protein species found in the gel were plotted...
against subunit number, a linear relationship was obtained, and the number of the largest species was 10. This finding demonstrates that GTP cyclohydrolase I is a decamer.

Inhibitory Complex Formation

**Dose Dependence of GFRP for Its BH₄-dependent Inhibitory Action on GTP Cyclohydrolase I Activity**—To determine the amount of GFRP required to totally inhibit GTP cyclohydrolase I, we examined the effect of varying amounts of GFRP on the activity of a fixed amount of GTP cyclohydrolase I (0.23 μM) in the presence of a saturating concentration of BH₄ (Fig. 2). Because the $K_v$ value for the association between GFRP and GTP cyclohydrolase I is in the nanomolar range (1), the concentrations used were saturating for formation of the protein complex. As shown in Fig. 2, the activity of GTP cyclohydrolase I was inhibited in a GFRP dose-dependent manner and became totally inhibited when the GFRP concentration was above 0.25 μM. This result indicates that the same number of GFRP as GTP cyclohydrolase I in terms of subunits is necessary and sufficient for total inhibition of the enzyme activity. Because GFRP is a pentamer (11) and GTP cyclohydrolase I is a decamer as shown above, this finding indicates that two molecules of GFRP are required for the total inhibition of one molecule of GTP cyclohydrolase I.

**Protein Stoichiometry of the Inhibitory Complex Formation between GTP Cyclohydrolase I and GFRP**—The kinetic experiments suggested that two molecules of GFRP were associated with one molecule of GTP cyclohydrolase I. To physically determine the number of GFRP associated with GTP cyclohydrolase I, we used a gel filtration method by which the complex was separated from free GFRP.

It should be noted that, when each protein was separately filtered through the resin in the presence of either or both BH₄ and GTP (data not shown), both proteins eluted from the gel resin at the same volume as those in the absence of ligands (Fig. 3, A1 and A2), indicating that these ligands induced neither dissociation into subunits of smaller number nor further association of each multimer. Thus, GFRP and GTP cyclohydrolase I are stable in terms of subunit assembly under the gel filtration conditions used. These properties of the proteins made the gel filtration experiments feasible for assessing complex formation.

We titrated GTP cyclohydrolase I with GFRP in the presence of 20 μM BH₄ and 200 μM GTP. No protein complex was formed in the absence of BH₄ and GTP or even in the presence of either of the two ligands under these experimental conditions (data not shown). In the presence of BH₄ and GTP, by contrast, the peak height of GTP cyclohydrolase I increased gradually as the amount of GFRP added increased (Fig. 3A). The peak height increased continuously until the moles (subunit) of GFRP equaled those of GTP cyclohydrolase I and then leveled off. GFRP did not elute as a free form until the molar amount of GFRP exceeded that of GTP cyclohydrolase I.

These observed alternations of the two peaks in the chromatograms were more evident when the heights of both peaks were plotted against the amount of GFRP added (Fig. 4A1). When the number of GFRP subunits equaled that of GTP cyclohydrolase I subunits, GFRP saturated GTP cyclohydrolase I. Therefore, we concluded that two molecules of a pentameric GFRP were associated physically with one molecule of a decameric GTP cyclohydrolase I. This conclusion is consistent with the conclusion derived from the kinetic data described above. The elution position of the complex changed little from that of free GTP cyclohydrolase I; consequently, these two species were not distinguished on the chromatograms. Thus, the peak assigned to the complex in the plot contained both free GTP cyclohydrolase I and the complex between GFRP and GTP cyclohydrolase I, but this apparently does not affect the conclusion.

To further verify this conclusion, we measured by gel electrophoresis the amounts of each protein species that were contained in the peaks eluted at 7 min. As shown in Fig. 5, when proteins contained in the peak were separated by gel electrophoresis, we observed that the amount of GFRP contained in those fractions increased until it became equimolar with GTP cyclohydrolase I and then leveled off. To estimate the amounts of each protein species in the gel, we cut the stained protein bands out of the gel, eluted the dye bound to the proteins, and measured the amount of eluted dye by its absorbance. As shown in Fig. 4A2, the amount of GFRP associated with GTP cyclohydrolase I increased linearly and leveled off at the same molar amount as that of GTP cyclohydrolase I. This result is consistent with the conclusion derived from the chromatograms. Furthermore, we quantitated each protein species by comparing the amount of eluted dye from the samples with that from varying amounts of standard GFRP and GTP cyclohydrolase I. We observed that 1 mol of GFRP subunit was associated with each mole of GTP cyclohydrolase I subunit for the samples derived from the complex in which GFRP saturated GTP cyclohydrolase I (data not shown).

**Behavior of the Complex on Gel Filtration**—As described above, the elution volume of the complex from a TSK G3000SWXL column was almost the same as that of GTP cyclohydrolase I. Since the elution position of the complex from the column was close to the void volume, it was outside the given linear estimation of molecular masses. Thus, we performed gel filtration using Seprose 6 resins, which gave a straight line over the molecular mass range of 150–669 kDa. GTP cyclohydrase I and the complex were eluted from this column at close volumes, which corresponded to the relative molecular masses of 300 and 313 kDa, respectively, when compared with those of bovine thyroglobulin (669 kDa), sweet potato β-amylase (200 kDa), and yeast alcohol dehydrogenase (150 kDa). This observation suggests that the radius of gyration of GTP cyclohydrase I did not change even after two molecules of GFRP with a molecular mass of 50 kDa were bound to the enzyme. Considering that the shape of the corresponding E. coli GTP cyclohydrase I is a torus formed by face-to-face association of two pentamers with dimensions of 65 × 100 Å (17), we infer that the structure of the complex is such that one molecule of the GFRP pentamer binds to each of the two outer faces of the pentamer of GTP cyclohydrase I; in other words, GTP cyclohydrase I is sandwiched between
GFRPs. Thus, the complex might behave on gel filtration as though the apparent diameter was similar to that of GTP cyclohydrolase I.

**Dose Dependence and Specificity of BH₄ and GTP for Their Inducibility of Complex Formation**—By using the gel filtration method, we examined the effect of varying concentrations of BH₄ on complex formation between GFRP and GTP cyclohydrolase I in the presence of GTP. As shown in Fig. 6A, the complex was formed in a BH₄-dose-dependent manner. The median effective concentration (EC₅₀) of BH₄ was estimated from the graph to be 2.0 μM. This value is equal to the EC₅₀ value of BH₄ (2.0 μM) that was kinetically determined for the inhibition of GTP cyclohydrolase I activity at 100 μM GTP (11). This finding suggests that the physical association of GFRP with GTP cyclohydrolase I is tightly coupled to the inhibition of enzyme activity.

We also examined the effect of varying concentrations of GTP on protein complex formation in the presence of BH₄. As shown in Fig. 6B, the complex was formed in a GTP dose-dependent manner. The EC₅₀ value of GTP was estimated to be 26 μM.

We examined the ability of two BH₄-related compounds to induce formation of the protein complex at a fixed saturating concentration of GTP. The compounds examined were BH₂ and L-biopterin, which are oxidized forms of BH₄ and have no cofactor activity for the known BH₄-requiring enzymes. As shown in Fig. 6A, BH₂ induced complex formation, and its EC₅₀ value was 4.7 μM. Thus, BH₂ is half as effective as BH₄. Biopterin induced no complex formation even at a concentration of 50 μM.
Thus, more potent effects were observed for more reduced biotinylations. These results are consistent with our kinetic data (1) that showed that BH₂ inhibits the activity of GTP cyclohydrolase I with an EC₅₀ value of 4 μM, whereas biotin is totally ineffective at a concentration of 50 μM. These findings indicate again the close relationship between the functional association of GFRP and GTP cyclohydrolase I and the catalytic activity of the enzyme.

We also examined the ability of GTP-related compounds to induce complex formation in the presence of 8 μM BH₄. As shown in Fig. 6B, dGTP showed a similar inducibility compared with GTP with an EC₅₀ value of 20 μM. Thus, the deoxylation of the ribose moiety at the 2'-position had a slight effect on complex formation. GTP-Y-S also induced complex formation with an EC₅₀ value of 18 μM. GDP induced complex formation, and its EC₅₀ value was 67 μM; however, the maximum binding of GFRP to GTP cyclohydrolase I was estimated to be about half of that with GTP. This finding may indicate that only one molecule of GFRP associated with one molecule of GTP cyclohydrolase I in the presence of GDP under the experimental conditions. GMP did not induce complex formation at all, even at a concentration of 100 μM.

To determine the structural requirement of the base portion of nucleotide triphosphates for complex formation, we examined the ability of ATP, inosine 5'-triphosphate, and xanthine 5'-triphosphate to induce formation of the protein complex. These compounds did not induce complex formation at all at a concentration of 100 μM.

The conversion of GTP to dihydroneopterin triphosphate did not appear to be required for formation of the complex between GTP cyclohydrolase I and GFRP because the enzyme activity was almost completely inhibited at 8 μM BH₄. Furthermore, while GTP-Y-S and dGTP induced formation of the protein complex as shown above, we found that these molecules did not serve as substrate for GTP cyclohydrolase I at all. After incubating each of these nucleotides with the enzyme, we measured the amount of nucleotide remaining in the reaction mixture. No decrease in either nucleotide was found upon incubation with the enzyme. These findings indicate that no enzymatic conversion of guanine nucleotides is involved in the protein complex formation.

Stimulatory Complex Formation

Dose Dependence of GFRP for Its Phenylalanine-dependent Stimulatory Action on GTP Cyclohydrolase I Activity—In the presence of GFRP, phenylalanine changes the kinetic nature of GTP cyclohydrolase I from sigmoidal to hyperbolic in a dose-dependent manner; in other words, phenylalanine stimulates the enzyme activity when GTP concentrations are below saturation (1, 11). To determine the amount of GFRP required for producing the maximum effect of phenylalanine, we examined the effect of varying concentrations of GFRP on the activity of GTP cyclohydrolase I at a non-saturating GTP concentration of 20 μM in the presence of 1 mM phenylalanine. With this phenylalanine concentration, the substrate velocity curve for GTP cyclohydrolase I fully changes from sigmoidal to hyperbolic (1, 11). The concentration of GTP used (20 μM) is a concentration at which the activity of GTP cyclohydrolase I increases ~4-fold in the presence of a saturating amount of GFRP and 1 mM phenylalanine (11). Under these conditions, GFRP stimulated the activity of GTP cyclohydrolase I in a dose-dependent manner and leveled off at a concentration of 0.2 μM, which is the same concentration as that of GTP cyclohydrolase I contained in the reaction mixture (Fig. 7). This result indicates that, as in the case of the GFRP-dependent inhibition of GTP cyclohydrolase I, an equimolar number of GFRP subunits is necessary for the total activation of GTP cyclohydrolase I activity, indicating that two molecules of GFRP are required for the total activation of one molecule of GTP cyclohydrolase I.

Protein Stoichiometry of the Stimulatory Complex Formation between GTP Cyclohydrolase I and GFRP—To physically determine the protein stoichiometry of the stimulatory complex formed between GFRP and GTP cyclohydrolase I, we performed similar gel filtration experiments as for the inhibitory complex. When each protein was separately filtered through the gel in the presence of phenylalanine, both GFRP and GTP cyclohydrolase I eluted from the gel resin at the same volumes as they did in the absence of any ligands, indicating that phenylalanine did not alter the association state of the subunits of either protein. This fact was a prerequisite for the following experiments.

With regard to the phenylalanine-dependent complex formation, we obtained chromatograms (Figs. 3B and 4A1) that were similar to those obtained for the inhibitory complex (Figs. 3A and 4A1). It should be noted that the increase in the peak height of GTP cyclohydrolase I was not a linear function of the amount of GFRP (Fig. 4B1). However, the quantitative analysis of proteins in the peak fraction by gel electrophoresis showed that the amount of GFRP associated with GTP cyclohydrolase I increased linearly and leveled off at the same molar amount as that of GTP cyclohydrolase I (Fig. 4B2). These findings indicate that phenylalanine also induces association of two molecules of pentameric GFRP with one molecule of decameric GTP cyclohydrolase I.

Dose Dependence and Specificity of Phenylalanine for Its Inducibility of Complex Formation—We examined the effect of varying concentrations of phenylalanine on complex formation. As shown in Fig. 8, the resulting curve was sigmoidal, indicating a positive cooperativity of phenylalanine in its ability to induce the association between GFRP and GTP cyclohydrolase I. By assuming that the binding of phenylalanine to the proteins induces formation of the protein complex, we treated the saturation data as a binding curve and converted it into a Hill plot: log[ΔA/(1 – ΔA)] versus log[phenylalanine], where ΔA was the measured change in the amount of GFRP bound to GTP cyclohydrolase I. The resulting plot was linear in its middle portion with a slope nₜ (Hill coefficient) of 2.0. The Kₘₜ of phenylalanine was estimated from the graph to be 65 μM.

Thus, a positive cooperative relationship between phenylalanine and complex formation was revealed. We then investigated the relationship between phenylalanine and enzyme activity in the presence of GFRP at 20 μM GTP, at which concentration the activity of GTP cyclohydrolase I is stimulated by phenylalanine in the presence of GFRP (1, 11). Interestingly, the curve of the enzyme activity plotted against the
phenylalanine concentrations was also sigmoidal (Fig. 9). When the saturation data were converted into a Hill plot, we obtained a slope \( n_H \) of 1.8 and a \( K_{0.5} \) of 90 \( \mu \)M. These values were comparable to those obtained from the above experiments on the phenylalanine-induced complex formation between GFRP and GTP cyclohydrolase I. These findings suggest a close relationship between the physical association of GFRP and GTP cyclohydrolase I and the changes in the kinetic properties of GTP cyclohydrolase I.

In contrast to L-phenylalanine, neither D-phenylalanine nor tyrosine induced complex formation at a concentration of 1 mM. We observed earlier that the latter two aromatic amino acids did not affect the activity of GTP cyclohydrolase I as L-phenylalanine did (1). Thus, the effect of the amino acid was highly specific to L-phenylalanine in terms of enzyme activation and complex formation.

**Conclusion**—We presented two lines of evidence, kinetic as well as physicochemical, demonstrating that two molecules of GFRP physically bind to one molecule of GTP cyclohydrolase I. The protein stoichiometry turned out to be the same for both types of protein complex induced by phenylalanine or by a combination of BH4 and GTP. Because GFRP is a pentamer (11) and GTP cyclohydrolase I is a decamer as shown by the cross-linking experiments presented in this paper, the complex thus contains equal numbers of subunits of GFRP and GTP cyclohydrolase I. This fact is consistent with the model we previously proposed regarding the structure of the protein complex, partly by assuming the general symmetric structure of protein complexes (11). In that model, we proposed that one molecule of GFRP binds to each face of a disc-like structure of GTP cyclohydrolase I, which is formed by face-to-face association of two pentamers. Such a structure of GTP cyclohydrolase I was revealed by x-ray crystallographic analysis of *E. coli* GTP cyclohydrolase I (17). Because the *E. coli* and rat enzymes exhibit considerable sequence similarity and have the same number of subunits, it is likely that rat GTP cyclohydrolase I has a three-dimensional arrangement of subunits similar to that of the bacterial enzyme.

The specificity of the biopterins and aromatic amino acids with regard to induction of protein complex formation was...
similar to that for the catalytic effects, *i.e.* BH₄ and phenylalanine were the most effective. Interestingly, the ability of nucleotides to induce protein complex formation was highly specific in terms of the structure of the base, but was nonspecific in terms of the hydroxylation state of the ribose moiety at the 2'-position and of the structure of the third position of phosphate.

Positive cooperativity was found with respect to phenylalanine in the association of GFRP with GTP cyclohydrolase I and also in the GFRP-mediated stimulation of GTP cyclohydrolase I activity, whereas no cooperative phenomenon was observed for either BH₄ and GTP in the inhibitory complex formation. The positive cooperativity means that GTP cyclohydrolase I activity increases sharply with only a relatively small increase in phenylalanine concentration. This function may operate physiologically as BH₄ is sufficiently supplied to phenylalanine hydroxylase when the plasma phenylalanine concentration increases.

Finally, when all the physical data presented herein on protein complex formation were compared with the corresponding kinetic data, a close relationship was found between the physical association of GFRP with GTP cyclohydrolase I and the change in the activity of GTP cyclohydrolase I. It is thus established that the regulation of GTP cyclohydrolase I by GFRP occurs through a mechanism of protein complex formation.

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