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23. We thank K. Farrell for discussions regarding the PelC similarities with tubulin and the Academic Computing Graphics and Visual Imaging Lab, University of California, Riverside, for help generating Fig. 5. Supported by U.S. Department of Agriculture award 91-37303-6645 and the San Diego Supercomputer Center.

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Feedback Regulation Mechanisms for the Control of GTP Cyclohydrolase I Activity

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Guanosine triphosphate (GTP) cyclohydrolase I, the rate-limiting enzyme in the biosynthesis of tetrahydrobiopterin (BH_4), is subject to feedback inhibition by BH_4, a cofactor for phenylalanine hydroxylase. Inhibition was found to depend specifically on BH_4 and the presence of another protein (p35). The inhibition occurred through BH_4-dependent complex formation between p35 protein and GTP cyclohydrolase I. Furthermore, the inhibition was specifically reversed by phenylalanine, and, in conjunction with p35, phenylalanine reduced the cooperativity of GTP cyclohydrolase I. These findings also provide a molecular basis for high plasma BH_4 concentrations observed in patients with hyperphenylalaninemia caused by phenylalanine hydroxylase deficiency.

The enzyme GTP cyclohydrolase I (E.C. 3.5.4.16) catalyzes the conversion of GTP to dihydroneopterin triphosphate, the first step of BH_4 biosynthesis in animals (*1*). One function of BH_4 is as a cofactor for phenylalanine hydroxylase as well as for tyrosine hydroxylase, tryptophan hydroxylase, the O-alkylglycerolipid cleavage enzyme (*1*), and nitric oxide synthases (*2*). In contrast to the other cofactors, BH_4 is one of the regulators of these enzymic reactions (*3*) and is thus implicated in the control of phenylalanine catabolism and of neural and immune functions. The intracellular BH_4 concentration is controlled by the rate of its de novo biosynthesis (*1*), mainly at the step catalyzed by GTP cyclohydrolase I. This enzyme is the rate-limiting enzyme in the synthetic pathway, and it has been suggested that its activity is regulated at transcriptional (*4*) and substrate (*5*, *6*) levels. The activity of GTP cyclohydrolase I in the liver is thought to be regulated by feedback inhibition. The enzyme activity in crude rat liver extracts is inhibited by BH_4 (*7*), and the in vivo occurrence of such feedback inhibition is strongly suggested by clinical observation of patients with an inherited disorder of BH_4 metabolism (*8*). However, the biochemical mechanisms underlying the feedback inhibition of this enzyme by BH_4 remain obscure.

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In the course of our studies on GTP cyclohydrolase I (*5*, *6*, *9*), we noticed that rat GTP cyclohydrolase I expressed in *Escherichia coli* (*10*) was not inhibited by BH_4. The recombinant rat enzyme could not be distinguished from the enzyme purified from rat liver on the basis of native molecular weight, subunit molecular weight, or ion spray mass spectrometric analysis of the molecular weights of peptides obtained from lysyl-endopeptidase digests of the two enzymes. Consistent with this finding, we determined that GTP cyclohydrolase I purified from rat liver was also not inhibited by BH_4, although the enzyme activity in crude liver extracts was inhibited, as reported (*7*). In order to determine if a dissociable inhibitory factor existed, we fractionated the 100,000*g* supernatant obtained from rat liver homogenates by gel filtration. Endogenous GTP cyclohydrolase I was no longer inhibited by BH_4 (Fig. 1, triangles), in sharp contrast to the inhibition of enzyme activity noted in the crude extracts. We found that the fractions that were eluted around a volume of about 88 ml inhibited GTP cyclohydrolase I activity in a BH_4-dependent manner (Fig. 1). The inhibitory factor was sensitive to digestion with proteinase K and trypsin and was insensitive to deoxyribonuclease I and ribonuclease, suggesting that it was a protein. We combined the fractions containing the inhibitory protein and used them for further characterization of this protein, which was designated as the p35 fraction according to its elution volume corresponding to a molecular size of 35 kD on Superdex 75 HR column chromatography (*11*).

The p35 fraction was incubated with recombinant GTP cyclohydrolase I. The reaction was linear up to 60 min both in the presence and in the absence of BH_4. In the presence of BH_4, the p35 fraction inhibited GTP cyclohydrolase I activity in a dose-dependent manner, but no inhibition was observed in the absence of BH_4. On the basis of these findings, we estimated that the total inhibitory activity contained in the p35 fraction derived from 1.0 g of liver was capable of inhibiting GTP cyclohydrolase I activity by 59 nmol/hour. Because the total GTP cyclohydrolase I activity recovered from the same amount of liver tissue was 10 nmol/hour, rat liver contained enough inhibitory activity to totally inhibit the activity of GTP cyclohydrolase I.

We examined the specificity of the inhibition of GTP cyclohydrolase I activity by p35 (*12*). A median effective concentration (EC_{50}) of BH_4 for the inhibition of GTP cyclohydrolase I by p35 was 2 μM at a GTP concentration of 0.1 mM, and the effect of BH_4 reached its maximum at about 7 μM. This range of effective concentrations is very similar to the hepatic concentration of BH_4, which was estimated to be approximately 6 μM from the reported value of 1.6 ± 0.4 μg per gram (wet weight) of rat liver (*13*), and

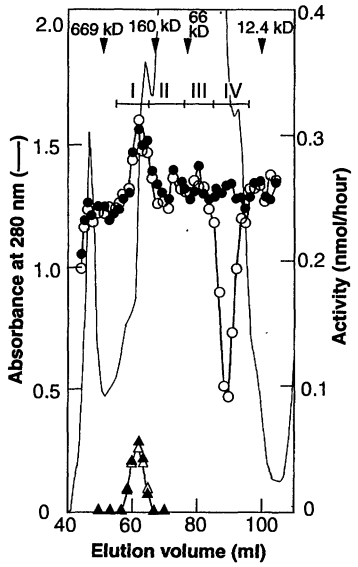

Fig. 1. Gel filtration chromatography data of the 100,000*g* supernatant of rat liver homogenates (*28*). Plotted are endogenous GTP cyclohydrolase I activity in the presence (△) or absence (▲) of 20 μM BH_4 and inhibitory activity in the presence (○) or absence (●) of 20 μM BH_4. The fractions were combined as indicated by the Roman numerals. Fraction IV (p35 fraction) was concentrated nearly tenfold. Calibration standards: thyroglobulin, 669 kD; γ-globulin, 160 kD; bovine serum albumin, 66 kD; cytochrome c, 12.4 kD.

this value corresponds to nearly the lowest BH_4 concentration at which GTP cyclohydrolase I activity is fully inhibited. Hence, when BH_4 decreases slightly below basal levels, the repression of its biosynthesis would be immediately released.

The p35 fraction and BH_4 inhibited GTP cyclohydrolase I by decreasing its maximum velocity (V_{max}) while having little effect on its affinity for GTP, indicating a noncompetitive type of inhibition (Fig. 2A). The extent of the inhibition was nearly constant above a GTP concentration of about 20 μM (Fig. 2A). Below this concentration, however, the inhibition gradually diminished with a decrease in the GTP level (Fig. 2A). This effect of GTP at lower concentrations may also contribute to the maintenance of a fixed BH_4 concentration in vivo; that is, when the intracellular concentration of GTP decreases below about 20 μM, the catalytic rate of this enzyme will decrease (6), but its inhibition by p35 and BH_4 will be weakened simultaneously (*14*).

Because the primary hepatic function of BH_4 is as a cofactor for phenylalanine hydroxylase, we examined the effect of phenylalanine on the inhibition of GTP cyclohydrolase I activity by p35 in the presence of varying concentrations of BH_4. Phenylalanine reversed the inhibition of GTP cyclohydrolase I activity by p35 and BH_4 while increasing the EC_{50} value of BH_4 (Fig. 2B) (*15*). The EC_{50} value of phenylalanine at a physiological BH_4 concentration of 6 μM was estimated to be 150 μM, which is similar to the hepatic phenylalanine concentration (reported to be 100 μmol per kilogram of liver weight) (*16*).

Because the inhibition by p35 was found to be reversible, we assessed possible complex formation between p35 and GTP cyclohydrolase I by gel filtration chromatography (*17*). When GTP and BH_4 were present in the equilibration buffer, the activity of GTP cyclohydrolase I was eluted in fraction I as in the control experiment, and the enzyme was inhibited by addition of BH_4 to the reaction mixture, indicating that p35 formed a complex with GTP cyclohydrolase I. On the other hand, when the incubation mixture and the equilibration buffer contained neither BH_4 nor GTP, or contained either one of them, the GTP cyclohydrolase I activity eluted in fraction I was not inhibited by addition of BH_4, indicating that both GTP and BH_4 were necessary for complex formation between p35 and the enzyme. The requirement of GTP for complex formation is consistent with the decrease in the inhibitory activity of p35 along with the decrease in the GTP concentration (Fig. 2A). When, to examine whether the complex was dissociated in the absence of GTP and

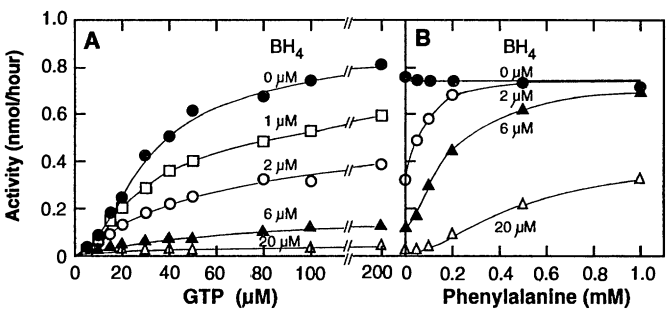

Fig. 2. (**A**) Effect of the BH_4 concentration on the substrate-velocity curve for GTP cyclohydrolase I in the presence of p35. The reaction was performed in a standard reaction mixture (*29*) containing 0.19 μg of recombinant GTP cyclohydrolase I, 30 μl of p35 fraction, and the specified concentrations of GTP in the presence of the indicated concentrations of BH_4. (**B**) Effect of phenylalanine concentration on BH_4-dependent, p35-mediated inhibition of GTP cyclohydrolase I. The reaction was performed in a standard reaction mixture (*29*) containing 0.19 μg of recombinant GTP cyclohydrolase I, 30 μl of the p35 fraction, 0.1 mM GTP, and the specified concentrations of phenylalanine in the presence of the indicated concentrations of BH_4.

BH_4, fraction I containing the complex was subjected to rechromatography on Superdex 200pg gel in buffer A, the inhibitory activity was shifted to fraction IV, and GTP cyclohydrolase I activity was recovered in fraction I and this activity was no longer inhibited in the presence of BH_4. These findings confirmed that the association and dissociation of p35 and GTP cyclohydrolase I occurred in the presence and absence of BH_4, respectively, providing that the GTP concentration was sufficient.

To investigate the mechanism through which phenylalanine reverses the inhibition of GTP cyclohydrolase I by p35 and BH_4, we examined the effect of phenylalanine on the binding of p35 to GTP cyclohydrolase I in the presence of BH_4. We carried out gel-filtration chromatography under the conditions noted above, except that phenylalanine was added to the incubation mixture and the equilibration buffer. The complex formed in the presence of phenylalanine, indicating that phenylalanine reversed the inhibitory action of p35 not through blocking its complex formation with GTP cyclohydrolase I but through changing the complex into some active form. Furthermore, we found that phenylalanine alone could induce complex formation between p35 and GTP cyclohydrolase I.

In the absence of p35, phenylalanine had no effect on the substrate-velocity curve of GTP cyclohydrolase I, but in the presence of p35 it changed the kinetic nature of the enzyme from sigmoidal to hyperbolic in a dose-dependent manner (Fig. 3). The Hill coefficient decreased from 1.85 to 1.0 in 10 mM phenylalanine. Phenylalanine thus stimulated enzyme activity in the presence of unsaturated GTP concentrations and showed no effect on V_{max}. This effect was also found to be specific to L-phenylalanine.

When the phenylalanine-induced enzyme complex in fraction I from the first chromatography was subjected to rechromatography, the GTP cyclohydrolase I activity recovered in fraction I was no longer stimulated by phenylalanine. The resulting fraction IV contained BH_4-dependent inhibitory activity as well as phenylalanine-dependent stimulatory activity for GTP cyclohydrolase I. Furthermore, so did fraction IV dissociated from the GTP and BH_4-induced complex. These results confirmed that the inhibitory and stimulatory activities were mediated through the same protein (p35) (*18*) (Fig. 4).

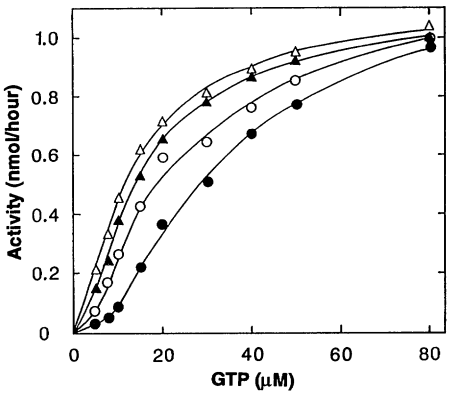

Fig. 3. Effect of phenylalanine concentration on the substrate-velocity curve for GTP cyclohydrolase I in the presence of p35. The reaction was performed in a standard reaction mixture (*29*) containing 0.19 μg of recombinant GTP cyclohydrolase I, 30 μl of the p35 fraction, the specified concentrations of phenylalanine [(●), 0 mM; (○), 0.1 mM; (▲), 1 mM; and (△), 10 mM], and varying concentrations of GTP.

When the hepatic phenylalanine concentration rises, for example, after dietary intake, phenylalanine is converted to tyrosine for further metabolism (*19*). Elevated concentrations of phenylalanine are known to activate phenylalanine hydroxylase by causing an increase in its V_{max} [for review, see (*20*)]. A high concentration of phenylalanine would simultaneously suppress the inhibition of GTP cyclohydrolase I by p35 and BH_4, thereby augmenting the amount of BH_4 available to act as a cofactor for activated phenylalanine hydroxylase; BH_4

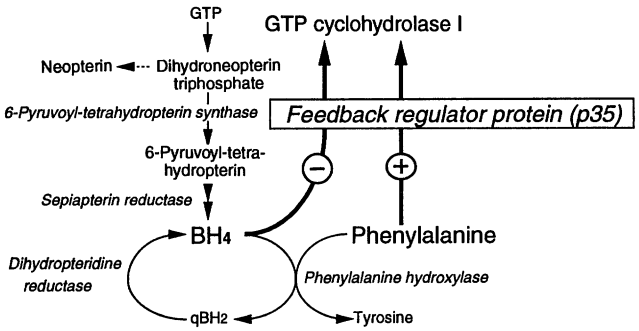

Fig. 4. Feedback regulation mechanisms of GTP cyclohydrolase I through feedback regulator protein by BH_4 and phenylalanine; BH_4 inhibits GTP cyclohydrolase I activity by inducing complex formation between the enzyme and feedback regulator protein. This "inactive" complex can be converted to an "active" form by phenylalanine. At low GTP concentrations, phenylalanine further alters the sigmoidal nature of this enzyme's kinetics to a hyperbolic pattern, consequently enhancing its activity. The activity of GTP cyclohydrolase I is dually controlled through feedback regulator protein by BH_4 (the end product) and by phenylalanine (the ultimate objective of this synthetic pathway).

is known to be a rate-limiting factor for phenylalanine hydroxylase (*21*). When the hepatic phenylalanine concentration falls, the body must repress the conversion of phenylalanine to tyrosine. A low concentration of phenylalanine has no substantial influence on the inhibition of GTP cyclohydrolase I by p35 and BH_4, which consequently prevents an unnecessary increase in the BH_4 concentration. Thus, p35 appears to behave as a supervisor of BH_4 biosynthesis, which reflects the current phenylalanine concentration, by regulating the rate-limiting step of the synthesis of this cofactor (Fig. 4). The activity of phenylalanine hydroxylase is directly regulated by phenylalanine and BH_4 (*22*), and thus the additional p35 regulation forms part of a complex system that facilitates the conversion of phenylalanine to tyrosine.

The phenylalanine regulatory mechanism for BH_4 biosynthesis revealed in this study accounts for several clinical observations related to phenylalanine metabolism, the underlying molecular mechanisms of which have long been unexplained, although these phenomena are well known and are even used in the diagnosis of related disorders. (i) Leeming *et al.* have reported that an oral phenylalanine load causes an increase in the plasma biopterin concentration in normal individuals (*23*). They also found that patients with hyperphenylalaninemia caused by phenylalanine hydroxylase deficiency had higher plasma biopterin concentrations than normal (*23*). Thus, high plasma phenylalanine concentrations induced an increase in the plasma biopterin concentration in both situations. (ii) Kaufman *et al.* have observed that, in patients with a partial defect in BH_4 biosynthesis, a phenylalanine load failed to increase the plasma biopterin concentration (*24*). They suggested that phenylalanine normally acts by stimulating one of the steps in BH_4 biosynthesis by some unknown mechanism. (iii) Furthermore, two clinical observations seem to point to the step catalyzed by GTP cyclohydrolase I as being stimulated by phenylalanine. McInnes *et al.* have reported a parallel change of plasma phenylalanine and neopterin concentrations in patients with a deficiency of 6-pyruvoyl-tetrahydropterin synthase (Fig. 4) (*25*). Dhondt *et al.* observed that an oral phenylalanine load increased the serum concentrations of both neopterin and phenylalanine, with the latter reaching 1 mM (*26*), a concentration capable of completely reversing the feedback inhibition of GTP cyclohydrolase I by p35 and BH_4. On the basis of these mechanisms, it would be interesting to reconsider the role of high plasma BH_4 concentrations and persistent activation of BH_4 biosynthesis in the neurological disorders that occur in phenylketonuria, the mechanisms of which have not been fully elucidated by hypotheses that attempted to ascribe the cause to high phenylalanine concentrations without taking into account the role of high BH_4 concentrations (*22*).

Our findings demonstrate regulation of the level of a cofactor by the substrate of an enzyme requiring that cofactor; this occurs through an alteration of the rate of biosynthesis of the cofactor itself and does not fit any of the categories of metabolic regulation proposed previously (*27*). We propose designating the protein p35 a "feedback regulator" protein for GTP cyclohydrolase I.

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8. In patients with BH_4-deficient hyperphenylalaninemia caused by a deficiency of 6-pyruvoyl-tetrahydropterin synthase, the enzyme that converts dihydroneopterin triphosphate to 6-pyruvoyl-tetrahydropterin, urinary excretion of neopterin (the degradation product of dihydroneopterin triphosphate) is increased [A. Niederwieser, H.-Ch. Curtius, M. Wang, D. Leupold, *Eur. J. Pediatr.* **138**, 110 (1982); S. Kaufman, G. Kapatos, R. R. McInnes, J. D. Schulman, W. B. Rizzo, *Pediatrics* **70**, 376 (1982); B. Beck, N. J. Brandt, E. Christensen, A. Niederwieser, P. S. Pedersen, *Acta Paediatr. Scand.* **72**, 449 (1983); A. Niederwieser *et al.*, *Eur. J. Pediatr.* **146**, 228 (1987)]. When BH_4 is administered to these patients, urinary neopterin excretion decreases in a dose-dependent manner, suggesting that BH_4 can inhibit GTP cyclohydrolase I activity in vivo.
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10. Rat GTP cyclohydrolase I cDNA (*9*) was expressed in *Escherichia coli* with the use of the pMAL vector (New England Biolabs). The resulting recombinant GTP cyclohydrolase I had the same molecular weight and NH_2-terminal sequence and could not be distinguished from the enzyme purified from rat liver.
11. An inhibitory activity was also identified in human liver. Both the rat and human p35 fractions inhibited either rat or human GTP cyclohydrolase I but did not inhibit *Escherichia coli* GTP cyclohydrolase I, implying that the effect of the inhibitory protein was mediated through a specific interaction with the eukaryotic enzymes.
12. The greatest facilitation of the inhibitory function was produced by 6*R*-BH_4 (referred to as BH_4 in the text), the naturally occurring and most effective cofactor. Its artificial enantiomer, 6*S*-BH_4, was more than one order of magnitude less effective than 6*R*-BH_4. In addition, 7-BH_4 [(7*R*,*S*)-5,6,7,8-tetrahydro-L-primapterin], which is an isomer of 6*R*-BH_4, was ineffective at a concentration of 50 μM. An analog of BH_4 with no side chain at the 6-position, PH_4, was more than one order magnitude less effective than 6*R*-BH_4. In contrast, two dihydropteridine compounds were severalfold less effective than 6*R*-BH_4. These were BH_2 (EC_{50} = 4 μM), an auto-oxidation product of 6*R*-BH_4, and sepiapterin (EC_{50} = 18 μM), a dihydro form of 6-lactoyl-tetrahydropterin (an immediate precursor of 6*R*-BH_4). The following pteridines were totally ineffective at a concentration of 50 μM: biopterin, 7-biopterin (L-primapterin), pterin, folate, dihydrofolate, and tetrahydrofolate. The following other reducing agents were ineffective at a concentration of 50 μM: NADH [reduced form of NAD^+ (nicotinamide adenine dinucleotide)], NADPH [reduced form of $NADP^+$ (nicotinamide adenine dinucleotide phosphate)], ascorbic acid, cysteine, and the reduced form of glutathione.
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14. In the presence of near physiological concentrations of p35, BH_4, and GTP, the activity of GTP cyclohydrolase I was completely inhibited within the examined enzyme concentration range of 0.5 to 20 nM (estimated at its native molecular size of 300 kD). The GTP cyclohydrolase I concentration in rat liver was estimated to be approximately 16 nM, from the calculated value of 4.84 μg/g (wet

weight) previously obtained by purification (*5*). Therefore, the inhibition of GTP cyclohydrolase I in rat liver appears to be fully dependent on the concentrations of p35, BH_4, and GTP. Dependence on the concentration of GTP cyclohydrolase I itself was also observed when the p35 concentration in the reaction mixture was reduced to one-fifth of its physiological concentration, and the EC_{50} of GTP cyclohydrolase I was 0.8 nM under such conditions.
15. This effect was highly specific to L-phenylalanine, and the following compounds were totally ineffective at a concentration of 1 mM: D-phenylalanine, β-phenylpyruvic acid, and phenethylamine (the immediate degradation products of phenylalanine), L-tryptophan and L-tyrosine (the substrates of tryptophan and tyrosine hydroxylase, respectively), and L-arginine (the substrate of nitric oxide synthases).
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17. The 100,000*g* supernatant of rat liver homogenates was incubated in buffer A [50 mM tris (pH 7.5), 100 mM KCl, and 1 mM EDTA, 1 mM dithiothreitol] containing GTP and BH_4 under conditions that completely inhibited GTP cyclohydrolase I activity. Then the mixture was filtered through Superdex 200pg gel in the equilibration buffers of various compositions. The eluates were collected into four fractions (Fig. 1), and each fraction was concentrated and filtered through a Sephadex G-25 column equilibrated with buffer A. Then each fraction was used for the assay of GTP cyclohydrolase I activity in the presence or absence of BH_4.
18. In immunoprecipitation experiments, similar results were obtained regarding the requirements for complex formation. Using a polyclonal antibody for the NH_2-terminal peptide nine amino acid residues of GTP cyclohydrolase I, we coprecipitated the p35 inhibitory and stimulatory activities with GTP cyclohydrolase I activity in the presence of both BH_4 and GTP or in the presence of phenylalanine. Thus, these experiments, in addition to the gel-filtration experiments, further confirmed the specific interaction of p35 with GTP cyclohydrolase I.
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21. Because the reported apparent Michaelis constants of phenylalanine hydroxylase for BH_4 are in the range of 2 to 15 μM [S. Kaufman and D. B. Fisher, in *Molecular Mechanisms of Oxygen Activation*, O. Hayaishi, Ed. (Academic Press, New York, 1974), pp. 285–369; R. Shiman, *Methods Enzymol.* **142**, 17 (1987)] and the intracellular BH_4 concentration in the liver is about 6 to 8 μM (*13*), this cofactor has been suggested to be one of the rate-limiting factors for phenylalanine hydroxylase. Moreover, we calculated the hepatic concentration of phenylalanine hydroxylase as 6.8 μM, using the reported values for the enzyme's specific activity in crude extracts and as a purified preparation as well as its reported subunit molecular weight [R. Shiman, D. W. Gray, A. Pater, *J. Biol. Chem.* **254**, 11300 (1979)]. This concentration is, surprisingly, nearly equal to that of BH_4, and therefore the hepatic BH_4 content is just enough for one-to-one binding to phenylalanine hydroxylase. In support of this hypothesis, it has been demonstrated that BH_4 restricts the conversion of phenylalanine to tyrosine in the hepatocytes (*19*).
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28. Rat liver was homogenized as described (*5*). The supernatant was separated from the residue by centrifugation at 100,000*g* for 30 min and was concentrated twofold by Amicon Centriflo CF25 ultrafiltration. An aliquot (2.5 ml) was applied to a Superdex 200pg column (1.6 cm by 60 cm) (Pharmacia) and eluted with 50 mM tris (pH 7.5), 100 mM KCl, and 1 mM EDTA at 1.0 ml/min. Eluates were monitored for absorbance at 280 nm. Fractions of 1.5 ml were collected, 45 μl of which was assayed for GTP cyclohydrolase I activity and for inhibitory activity in the presence of recombinant GTP cyclohydrolase I (0.1 μg) (*29*).
29. The activity of GTP cyclohydrolase I was assayed as described (*5*). The standard reaction mixture (50 μl) contained 50 mM tris (pH 7.6), 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM GTP, and the enzyme. The reaction was carried out at 37°C for 15 min. Then the reaction product, dihydroneopterin triphosphate, was converted to neopterin by oxidation with I_2 and KI, and dephosphorylation by alkaline phosphatase was performed as described (*5*). Finally, neopterin was quantitated by high-performance liquid chromatography and fluorescent detection.
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Antisense Gene Inhibition by Oligonucleotides Containing C-5 Propyne Pyrimidines

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Phosphorothioate oligodeoxynucleotides containing the C-5 propyne analogs of uridine and cytidine bind RNA with high affinity and are potent antisense inhibitors of gene expression. In a cellular assay, gene-specific antisense inhibition occurred at nanomolar concentrations of oligonucleotide, was dose-dependent and exquisitely sensitive to sequence mismatches, and was correlated with the melting temperature and length of oligonucleotide. Activity was independent of RNA target site and cell type but was detectable only when the oligonucleotides were microinjected or delivered with cell-permeabilizing agents. These oligonucleotides may have important applications in therapy and in studies of gene function.

Oligodeoxynucleotides (ODNs) offer great promise as antisense agents for the sequence-specific inhibition of gene expression (*1*). Previously, high concentrations of ODNs have been required to elicit biological effects, frequently leading to nonspecific inhibition of gene expression (*2*, *3*). There has rarely been a direct demonstration of ODNs entering cells and blocking protein expression through sequence-specific RNA interactions. In general, the deficiencies of antisense assays fall into one or more of the following categories: (i) indirect measurement of protein production, (ii) failure to measure the synthesis of a nontargeted protein as an internal control, and (iii) omission of mismatch and scramble-sequence controls.

Here we demonstrate sequence-specific and gene-specific antisense inhibition by C-5 propyne–substituted phosphorothioate ODNs in a reproducible and controlled assay. This assay uses microinjection and rapid protein analysis to compensate for poor cellular uptake (*1*) and rapid intracellular degradation (*4*) of unmodified ODNs. Plasmids that direct the expression of two target reporter genes, SV40 large T antigen (TAg) and *Escherichia coli* β-galactosidase (β-gal) together with antisense ODNs, were delivered to African Green monkey kidney (CV-1) or rat fibroblast (Rat2) cells by nuclear microinjection, and protein production was monitored by immunofluorescence (*5*). Co-injection of the two plasmids resulted in detectable amounts of each protein in the same cell at 4.5 hours after injection (*5*). A mutant TAg protein with a $Pro^{584} \rightarrow$ Leu substitution was used because it is expressed in greater amounts than the wild-type TAg (*6*) and therefore can be detected more readily. We chose an antisense target sequence located ~150 nucleotides (nt) downstream of the TAg translation initiation codon because this RNA site had been previously targeted with microinjected antisense RNA (*7*). The β-gal expression plasmid provided a control for cytostatic and nonspecific effects of the ODNs.

We studied the structure-activity relation of antisense inhibition by introducing phosphate, sugar, and pyrimidine modifications into ODNs (Fig. 1). These chemical

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