Atypical N-terminal Extensions Confer Novel Regulatory Properties on GTP Cyclohydrolase Isoforms in Drosophila melanogaster

Christopher D. Funderburk, Kevin M. Bowling, Dong Xu, Zhinong Huang, and Janis M. O’Donnell
From the Department of Biological Sciences, University of Alabama, Tuscaloosa, Alabama 35487

The cofactor tetrahydrobiopterin plays critical roles in the modulation of the signaling molecules dopamine, serotonin, and nitric oxide. Deficits in cofactor synthesis have been associated with several human hereditary diseases. Responsibility for the regulation of cofactor pools resides with the first enzyme in its biosynthetic pathway, GTP cyclohydrolase I. Because organisms must be able to rapidly respond to environmental and developmental cues to adjust output of these signaling molecules, complex regulatory mechanisms are vital for signal modulation. Mammalian GTP cyclohydrolase is subject to end-product inhibition via an associated regulatory protein and to positive regulation via phosphorylation, although target residues are unknown. GTP cyclohydrolase is composed of a highly conserved homodecameric catalytic core and non-conserved N-terminal domains proposed to be regulatory sites. We demonstrate for the first time in any organism that the N-terminal arms of the protein serve regulatory functions. We identify two different modes of regulation of the enzyme mediated through the N-terminal domains. The first is end-product feedback inhibition, catalytically similar to that of the mammalian enzyme, except that feedback inhibition by the cofactor requires sequences in the N-terminal arms rather than a separate regulatory protein. The second is a novel inhibitory interaction between the N-terminal arms and the active sites, which can be alleviated through the phosphorylation of serine residues within the N termini. Both mechanisms allow for acute and highly responsive regulation of cofactor production as required by downstream signaling pathways.

The pteridine (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄) is an essential cofactor for tyrosine hydroxylase (EC 1.14.16.3) (1), tryptophan hydroxylase (EC 1.14.16.4) (2), phenylalanine hydroxylase (EC 1.14.16.2) (3), glycerol ether monooxygenases (4, 5), and the various isoforms of nitric oxide synthase (6, 7). In addition to its redox function in the catalytic reactions of these enzymes, BH₄ has regulatory roles, because its cellular concentrations are often limiting. Changes in cellular pools of BH₄ therefore dictate the rate of production of key cell-signaling molecules, including dopamine, serotonin, and nitric oxide. The conversion of GTP to 2,4-dihydroneopterin triphosphate (H₂NTP), catalyzed by GTP cyclohydrolase I (GTPCH I, EC 3.5.4.16), is the first and rate-limiting reaction in the de novo synthesis of BH₄ in all higher eukaryotes (8). The importance of the proper regulation of GTPCH activity and BH₄ production is underscored by diseases associated with mutations in the BH₄ biosynthetic pathway. Autosomal dominant mutations in the GTPCH gene are associated with 3,4-dihydroxyphenylalanine-responsive dystonia (9–11), whereas autosomal recessive mutations are linked to hyperphenylalaninemia, an acute form of phenylketonuria (12). BH₄ deficiency has also been implicated in a variety of neurological disorders, including Parkinson disease (13), Alzheimer disease (14), and depression (15), as well as various vascular endothelial dysfunctions (16–18).

Mammalian GTPCH is regulated by a variety of mechanisms, the best characterized of which is end-product feedback inhibition by BH₄ (19). This inhibition involves the formation of a complex between GTPCH, GTPCH feedback regulatory protein (GFRP), and BH₄ (20, 21). The consequence of formation of this complex is a decrease in Vₘₐₓ via a mechanism that is non-competitive with the GTP substrate (19). In the presence of BH₄, one GFRP pentamer binds to each pentameric face of GTPCH, a homodecameric protein generated from the association of two pentameric toroids (22, 23). L-Phenylalanine reverses this inhibition (23). 2,4-Diamino-6-hydroxypteridine (DAHP), the prototypical inhibitor of GTPCH, has been widely used to modulate the activity of the enzyme and hence BH₄ production, to study the roles of GTPCH and BH₄ in physiologic pathways.

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1 The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S4 and Table S1.

2 We dedicate this paper to the memory of the late Edward Weisberg, whose pioneering doctoral research in Drosophila GTPCH biochemical analysis set the stage for the work reported here.

3 Both authors contributed equally to this work.

4 Present address: Center for Behavioral Neuroscience, Emory University School of Medicine, Atlanta, GA 30329.

5 Present address: Dept. of Pharmacology, School of Medicine, University of North Carolina, Chapel Hill, NC 27599.

6 The abbreviations used are: BH₄, (6R)-5,6,7,8-tetrahydro-L-biopterin; GTPCH, GTP cyclohydrolase; DAHP, 2,4-diamino-6-hydroxypteridine; GFRP, GTPCH feedback regulatory protein; TH, tyrosine hydroxylase; H₂NTP, dihydronopterin triphosphate; PKA, protein kinase A; PKC, protein kinase C; MUSCLE, multiple sequence comparison by log-expectation; HPLC, high pressure liquid chromatography; ANOVA, analysis of variance.
iological conditions and disease states (24, 25). The mechanism by which DAHP is able to inhibit GTPCH is similar to that of BH₄ in that GFRP is required for the inhibition and the inhibition is non-competitive (26, 27). DAHP, accordingly, has been used to induce BH₄ deficiencies in animal models of BH₄-deficient neurological disease (28) as well as hyperphenylalaninemia (29).

A second means of negatively regulating GTPCH activity appears to involve the expression of alternative isoforms. In humans, there are at least six alternatively spliced GTPCH mRNAs, with the translated proteins differing only in their C termini, yet only GTPCH type I is enzymatically active (30). Co-expression of GTPCH type I and GTPCH type II, a truncated and non-functional GTPCH protein, in human blood cells depresses the level of GTPCH type I protein (31). It remains unclear whether this mechanism is employed more universally for negative regulation of GTPCH in mammalian cells.

GTPCH is also positively regulated via post-translational modification. It has been reported that stimulation of GTPCH activity in mammalian cell culture occurs through phosphorylation and that GTPCH serves as a substrate for both casein kinase II and protein kinase C (32, 33). However, specific sites of phosphorylation have not yet been identified in mammalian GTPCH, nor has the effect of phosphorylation been defined enzymatically.

The sequence of GTPCH is highly conserved; the Drosophila melanogaster homolog is nearly 80% similar to the mammalian form. Moreover, its reaction mechanism appears to be conserved, as does the decameric structure (34). This high degree of conservation in a genetic model organism affords the opportunity to investigate, in greater detail, the mechanisms of regulation and their effects on the organism. Drosophila GTPCH is encoded by the gene Punch (Punch), which has been subjected to extensive genetic and molecular analysis (35–37). The Punch locus produces at least four transcripts by alternative promoters and alternative splicing. Three of these, transcripts A (1.70 kb), B (1.75 kb), and C (1.80 kb), are well characterized (38). Most of the predicted sequence for each of the resulting polypeptides is virtually identical to the catalytic core protein in mammals. The GTPCH polypeptides from all species analyzed to date have a non-conserved N-terminal domain that extends as an “arm” from the catalytic core. These arms have been variously proposed to be regulatory domains or docking sites for interacting proteins, although evidence is lacking to confirm these hypotheses. The Drosophila isoforms similarly have non-conserved N-terminal domains (supplemental Fig. S1). The isoforms, however, differ from the mammalian forms in three aspects. First, each has a unique N-terminal domain rather than differing at the C terminus as is the case for the mammalian protein. Second, the N termini are significantly longer than any other characterized GTPCH. Third, unlike the mammalian forms, which seem to be expressed in the same cells, the Drosophila isoforms are expressed in different tissues and at different times during development (36, 38, 39).

We have undertaken an in vitro analysis of recombinant Drosophila GTPCH isoforms to test for the first time the hypothesis that the N-terminal domains have regulatory functions. The studies reported here demonstrate that each of the Drosophila isoforms is catalytically active, and they provide evidence for a key regulatory region in the N termini of isoforms B and C. We also show that the N-terminal domains of these two isoforms serve as substrates for protein kinase A (PKA) and protein kinase C (PKC). Moreover, we obtained two surprising results. First, the distal residues in these arms strongly affect the kinetic properties of the enzymes, suggesting a heretofore unknown regulatory interaction between the arms and the active sites. Second, the N-terminal extensions serve as functional homologs of the mammalian GFRP despite the non-alignment of sequence, in that they are capable of directing non-competitive inhibition by BH₄ and DAHP in the absence of GFRP.

**EXPERIMENTAL PROCEDURES**

Cloning of Drosophila GTPCH Isoforms—Cloning of cDNAs corresponding to Drosophila GTPCH isoforms A and B was described previously (38). Isoform C cDNA was cloned from a 0- to 24-h Drosophila embryonic library (Stratagene, La Jolla, CA) containing cDNA clones in the bacteriophage λ ZAP using methods essentially the same as described previously (38).

PCR Generation and Subcloning of Proteins—To facilitate insertion of the various GTPCH constructs into the pQE30 expression vector (Qiagen), primers for PCR were designed to include the desired restriction enzyme recognition sites, with the various forward primers containing a site for BamHI digestion and the sole reverse primer containing a HindIII recognition site (5′-ACCAAGCTTTATTTGCTATTTAGACT-3′). PCR was performed by standard methods. Products of the appropriate size were observed and gel-purified (Stratagene).

For subcloning of Drosophila GTPCH isoforms A–C, the following forward primers were used: GTPCH A (5′-GACATTGG- ATCCAGGAGCCAAAGAC-3′); GTPCH B and GTPCH C (5′-CTCCAGATCCGCTCCAGCCCAACT-3′). For the common region, the forward primer (5′-GTACATTGGATCCCAAGAAAGTGCACGTTC-3′) was used. For the truncation analysis, the following forward primers were used: −30 isoform C (5′-CTCCAGATCCGCTCCAGCCCAACCAAG-3′) and −58 isoform C (5′-CTCCAGATCCGCTCCAGCCCAAACT-3′).

The N-terminal extensions were subcloned using the following reverse primers along with the appropriate forward primers: N-terminal isoform A (5′-CGGTTCAGTTGCTCCAGTTCCAATGTGCT-3′), N-terminal isoform B (5′-GTACATTGGATCCCAAGAAAGTGCACGTTC-3′), and N-terminal isoform C (5′-GTACATTGGATCCCAAGAAAGTGCACGTTC-3′). For site-directed mutagenesis of isoform C, the forward primer (5′-TCCAGGATCCATGAGCCTTTACCCGCCAAACT-3′) and the reverse primer (5′-GAACAAAGCTTTATTTGCTATTTAGACT-3′) were employed in conjunction with the following primers to incorporate the appropriate codon changes: S37A Forward (5′-CGGGAGACGACAAAAACCAAGCCGCTGCTCACAAGTAG-3′), S37A Reverse (5′-CTACATTGGGCGACAGGCGGCGGTTGTTGCCTCCGC-3′), S37E Forward (5′-CGGGAGACGACAAAAACCAAGCCGCTGCTCACAAGTAG-3′), and S37E Reverse (5′-CTACATTGGGCGACAGGCGGCGGTTGTTGCCTCCGC-3′).

Expression and Purification of Recombinant Proteins—PCR products were cloned into the pQE30 vector (Qiagen) for prokaryotic expression of His₆-tagged GTPCH by standard meth-
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ods. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen), and sequence confirmation analysis was performed by the Auburn University Genomics and Sequencing Laboratory. The verified DNA was used to transform competent M15 cells harboring the kanamycin-resistant pREP4 plasmid, which provides an inhibitor to expression. Transformed cells were grown on LB plates containing ampicillin (100 mg/liter) and kanamycin (25 mg/liter). A single colony was picked and grown in 1 liter of LB media containing ampicillin (100 mg/liter) and kanamycin (25 mg/liter). When bacterial density reached 0.6 at $A_{600}$, 1 ml isopropyl-1-thio-$eta$-D-galactopyranoside (LabScientific, Inc.) was added to induce recombinant protein expression. After 5 h at 37 °C, the bacterial culture medium was centrifuged for 20 min at 15,000 × g, and the resulting pellet was stored at −80 °C until use. The cell pellet was thawed for 20 min on ice and resuspended in 20 ml of lysis buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole, pH 8.0). Lysozyme (1 mg/ml) and one tablet of Complete protease inhibitor mixture (Roche Applied Science) were added, and the mixture was stirred on ice for 30 min. Bacteria were lysed by sonication, and the lysate was cleared by centrifugation at 80 °C until use. The cell pellet was maintained at 0.2 unit of recombinant mouse catalytic subunit PKA (pH 8.0, Calbiochem) or 10 ng of recombinant rat brain catalytic subunit PKC (pH 7.5, Calbiochem) and incubated at 30 °C for 30 min. GTPCH phosphorylation reactions were stopped by adding protein loading buffer and boiling for 10 min. Proteins were resolved by 15% SDS-PAGE. X-ray film was placed on the dried gel, exposed, and developed following standard methods.

Kinetic Microplate Assay for GTPCH Activity—Formation of H$_2$NTP from GTP (Roche Applied Science) was monitored using a kinetic microplate assay for GTPCH activity (27, 44). An increase in $A_{340}$ at 15-s intervals over a 1-h period at 37 °C was used to measure the accumulation of H$_2$NTP using a Thermomax microplate spectrophotometer (Molecular Devices). All reaction mixtures were 200 μl (final volume) and contained 100 mM Na$^+$/K$^+$ phosphate buffer (93.5 mM Na$_2$HPO$_4$, 6.5 mM KH$_2$PO$_4$, pH 7.8) or 50 mM Tris-HCl buffer (50 mM Tris, 300 mM NaCl, 0.1% Tween 20, 10% glycerol, pH 7.5), 0.05 μM GTPCH, and various concentrations of GTP and DAHP. A standard curve was generated by complete enzymatic conversion of desired concentrations of GTP to H$_2$NTP by 0.2 μM GTPCH isoform C as described (27, 44).

HPLC-based Assay for GTPCH Activity—GTPCH activity was assayed by a modification of a method described previously (45). All reaction mixtures were 70 μl (final volume) and contained 0.2 μM purified GTPCH, 0.25 mM GTP (Roche Applied Science), and various concentrations of BH$_4$, in either 100 mM Na$^+$/K$^+$ phosphate buffer or 50 mM Tris buffer. Reactions were incubated in the dark at 37 °C for 60 min. The reaction was stopped, and the product, H$_2$NTP, was oxidized to neopterin triphosphate by adding 30 μl of a 1% I$_2$/2% KI solution in 1 M HCl and incubating at 37 °C for 60 min in the dark. Samples were decolorized with 15 μl of 3% ascobic acid and centrifuged for 5 min at 10,000 × g. The samples were neutralized with 25 μl of 1 M NaOH and dephosphorylated at 37 °C for 30 min in a 70-μl mixture consisting of 50 μl of the oxidized neopterin triphosphate mixture, 2 units of calf intestinal alkaline phosphatase (Roche Applied Science), 7 μl of 10× dephosphorylation buffer (Roche Applied Science), 3 μl of 1 M NaOH, and 8 μl of H$_2$O. Neopterin was then quantified by reverse-phase HPLC following centrifugation through micro-spin centrifuge filter tubes (Alltech) with 0.2-μm pore size. Chromatographic separations were performed on an ESA CoulArray (Model 5600A) HPLC instrument. The mobile phase contained 75 mM sodium phosphate adjusted to pH 3.0 with phosphoric acid, 0.75 mM octanesulfonic acid, 25 μM EDTA, 100 μl/liter triethyleneglycol, and 7% acetonitrile. Separations were performed on a Phenomenon Synergi 4-μm Hydro-RP (4.6 × 15 cm) column, preconditioned with 500 ml of buffer before use and run with an isocratic flow at 0.5 ml/min. Neopterin was detected by fluorescence excitation at 360 nm/emission 465 nm with a Linear Model LC305 fluorescence detector. Neopterin content was determined by comparison with commercial neopterin (Sigma) as standard using ESA CoulArray software.

PKA Treatment and GTPCH Activity Analysis—Recombinant GTPCH isoforms B and C were expressed and lysed as above. A 45% ammonium sulfate precipitation was performed by dropwise addition of saturated ammonium sulfate solution to the cleared cellular lysate. Following incubation on ice for 1 h, the mixture was centrifuged 10,000 × g for 15 min. The protein pellet, containing GTPCH, was resuspended in 4.5 ml of kinase buffer (50 mM Tris-HCl, 10 mM MgCl$_2$, 200 μM ATP, pH 7.5). Treatment or control samples of the resulting mixture (2 ml) were treated with either 5 kilounits of recombinant mouse catalytic subunit PKA (Calbiochem) or equal volume kinase buffer, respectively. The samples were incubated at 30 °C for 30 min. A 50% slurry of nickel-nitriolitriacetic acid resin (500 μl, Qiagen) was added to each sample, and purification of GTPCH was performed as above, except that the protein was eluted with 200 μl of buffer. Proteins (0.05 μM) were assayed in 100 mM Na$^+$/K$^+$ phosphate buffer for enzymatic activity using the kinetic microplate assay.
**RESULTS**

**Kinetic Analysis of Recombinant Drosophila GTPCH Isoforms**—To investigate the kinetic properties of Drosophila GTPCH isoforms A–C, recombinant proteins were purified from an *Escherichia coli* expression system (supplemental Fig. S2). Purified isoforms A–C consistently exhibit higher molecular masses on SDS-PAGE (39, 45, and 47 kDa, respectively) than the calculated molecular masses (30, 34, and 36 kDa, respectively) as noted previously for *Drosophila* (38), rat (46), and human (47, 48) GTPCH subunits on SDS-PAGE. This discrepancy appears to stem, in part, from the N-terminal extensions of isoforms A–C, which exhibit apparent higher molecular masses (16, 17, and 18 kDa, respectively) than expected (8, 12, and 13 kDa, respectively), whereas the mobility of the common region is consistent with its expected molecular mass at 24 kDa.

The recombinant isoforms were assayed for enzymatic activity using a kinetic microplate assay (27, 44). Unlike the mammalian splice variants (30), all three *Drosophila* alternative isoforms exhibit enzymatic activity (Table 1). The reported \( K_{\text{m}} \) and \( V_{\text{max}} \) values for the three isoforms are consistent with previously published values for purified *Drosophila* (34) and mammalian GTPCH enzymes (26, 27, 49, 50). Inhibition by GTP substrate was also noted for all three isoforms at substrate concentrations exceeding 0.5 mM GTP (data not shown), which parallels previous observations with purified *Drosophila* GTPCH (34). Interestingly, a marked difference was noted in \( V_{\text{max}} \) between isoform A and isoforms B and C in both Tris and phosphate buffers, with isoform A exhibiting a \( V_{\text{max}} \) approximately twice that of isoforms B and C. Isoforms B and C, which differ only in a 16-amino acid addition to the N-terminal domain of isoform C, exhibit similar activity. The differences noted between isoforms A and isoforms B and C can be attributed to the N-terminal domain of isoform A, as it differs completely in sequence from those of isoforms B and C.

In accordance with previous observations of *Drosophila* GTPCH purified from crude head extracts, all three recombinant enzymes show positive cooperativity with Hill numbers exceeding 1.0 in Na\(^+\)/K\(^+\) phosphate buffer. In Tris buffer, marked positive cooperativity was noted for isoforms A and B, but not for isoform C. This observation is consistent with a buffer difference in positive cooperativity reported previously for GTPCH purified from adult *Drosophila* (34).

**Drosophila GTPCH Isoforms Are Subject to Feedback Inhibition by BH4**—Mammalian GTPCH is subject to feedback inhibition by BH4 only in the presence of GFRP (19). To determine whether recombinant *Drosophila* GTPCH was similarly unresponsive to BH4, each purified isoform was assayed for activity in the presence of various concentrations of BH4 by HPLC analysis (Fig. 1). Surprisingly, all three isoforms show inhibition by BH4 (supplemental Fig. S3). In Tris buffer, enzymatic activity was nearly abolished by 1 \( \mu \text{M} \) BH4. This BH4 concentration had little effect on GTPCH in phosphate buffer. However, even in phosphate buffer, BH4 strongly inhibited GTPCH activity at concentrations of 20 \( \mu \text{M} \) or higher. This inhibition was unexpected because GFRP is not present in the *Drosophila* genome;

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**TABLE 1**

Kinetic analysis of *D. melanogaster* GTPCH isoforms

| Isoform | \( K_{\text{m}} \) (\( \mu \text{M} \)) GTP | \( V_{\text{max}} \) (nmol NTP/min/mg protein) | Hill number | \( K_{\text{m}} \) (\( \mu \text{M} \)) BH4 | \( V_{\text{max}} \) (nmol NTP/min/mg protein) | Hill number |
|---------|-----------------|-----------------|------------|-----------------|-----------------|------------|
| A       | 82.2 ± 7.1      | 45.45 ± 2.43    | 1.79 ± 0.28| 70.4 ± 5.3      | 60.89 ± 2.82    | 2.03 ± 0.34|
| B       | 103.5 ± 9.2     | 26.49 ± 1.52    | 1.89 ± 0.27| 95.3 ± 9.3      | 29.07 ± 1.91   | 2.13 ± 0.42|
| C       | 75.3 ± 15.2     | 15.19 ± 1.61    | 1.30 ± 0.33| 76.0 ± 4.3      | 27.28 ± 0.86   | 1.45 ± 0.12|

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**FIGURE 1.** Effect of BH4 on recombinant GTPCH activity. GTPCH (0.2 \( \mu \text{M} \)) activity was assayed using the HPLC-based method described under “Experimental Procedures” with various concentrations of BH4 in Tris and Na\(^+\)/K\(^+\) phosphate buffers. Data shown are for GTPCH isoform B. Values are means ± S.E. of four determinations. **, significant difference from neopterin content at 0 \( \mu \text{M} \) BH4 in the respective buffer, \( p < 0.01 \) (one-way ANOVA with Dunnett post test).
nevertheless, the Drosophila recombinant enzymes exhibited robust inhibition comparable to mammalian GTPCH with GFRP.

GFRP-independent Inhibition of Drosophila GTPCH Isoforms by DAHP Is Non-competitive and Requires N-terminal Extensions—To further characterize this surprising inhibition of Drosophila GTPCH, we employed the prototypical GTPCH inhibitor, DAHP, in activity assays over a range of GTP concentrations (Fig. 2, A–C). All three isoforms exhibit non-competitive inhibition in the presence of DAHP with decreasing $V_{\text{max}}$ and unchanging $K_{0.5}$ as inhibitor concentration increased. As with $\text{BH}_4$, the inhibition of the Drosophila enzymes by DAHP does not require GFRP. Nevertheless, this non-competitive inhibition is remarkably similar in nature to that observed with mammalian GTPCH in the presence of $\text{BH}_4$ or DAHP and GFRP (19, 27).

Because the common region of Drosophila GTPCH is nearly identical to the mammalian protein, we hypothesized that the unique N-terminal extensions must facilitate the non-competitive inhibition, functionally replacing the mammalian regulatory protein. To test this hypothesis, we generated N-terminally truncated Drosophila GTPCH isoforms. We tested a truncated isoform C, lacking the first 58 amino acid residues ($\sim 58$ isoform C truncation), as well as a protein lacking any N-terminal extension (common region, $\sim 116$ amino acid residues for isoform C). The $\sim 58$ isoform C truncation displayed non-competitive inhibition in the presence of DAHP similar to that of the full-length enzymes (Fig. 2D). In contrast, removal of the entire N-terminal extension resulted in the inability of DAHP to inhibit the enzyme (Fig. 2E). These results suggest that the unique N-terminal extensions are indeed responsible for the non-competitive inhibition. Moreover, for isoform C, amino acid residues 59–116 appear to confer this regulatory property.

DAHP Inhibits Drosophila GTPCH in Vivo—In mammals, DAHP is capable of inhibiting GTPCH in vivo (24, 25). In mammalian cells, GFRP is required to facilitate this inhibition (26). Recently, inhibition of swallowtail butterfly, Papilio xuthus, GTPCH by DAHP has also been demonstrated (51). To eliminate the possibility that the DAHP inhibition observed is an in vitro artifact, we tested whether DAHP is capable of inhibiting Drosophila GTPCH in vivo. To this end, 72 h post-eclosion, male Drosophila were fed either DAHP (10 or 50 mM) in 5% sucrose/5% Me$_2$SO or 5% sucrose/5% Me$_2$SO alone (control) for 72 h. Whole fly extracts were subsequently assayed for $\text{BH}_4$ by HPLC (Fig. 3A). BH$_4$ levels decreased with DAHP ingestion, consistent with GTPCH inhibition in vivo. We noted a potential threshold inhibition level for 72-h DAHP administration, with low variation between 10 and 50 mM DAHP treatments. Following the removal of Drosophila from DAHP-containing media, BH$_4$ pools returned to wild-type levels (Fig. 3B), suggesting that, in Drosophila, DAHP acts as a classic enzymatic inhibitor rather than eliciting permanent alteration in GTPCH activity. Thus, DAHP inhibition of GTPCH occurs in vivo in

![FIGURE 2. Influence of DAHP on recombinant protein activity. Activity was assayed using the kinetic microplate assay as the quantity of H$_2$NTP produced by 0.05 μM recombinant protein over a range of GTP concentrations in Tris buffer using various DAHP concentrations (○, 0 mM; ■, 0.1 mM; ▲, 0.2 mM; ▼, 0.3 mM; ●, 0.5 mM; ◇, 0.7 mM DAHP). A, isoform A; B, isoform B; C, isoform C; D, $\sim 58$ isoform C truncation; E, common region. Nonlinear regression analysis of the data shows that inhibition by DAHP is non-competitive with decreasing $V_{\text{max}}$ with increasing inhibitor concentration ($K_{0.5}$ essentially unchanged) for A–D. No inhibition was detected for common region alone (E).]
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**Drosophila** as in mammals, despite the absence of a **Drosophila** GFRP homolog.

Truncation Analysis Reveals Native Negative Regulatory Region of **Drosophila** GTPCH—From the kinetic analysis of the three recombinant **Drosophila** GTPCH isoforms, it is evident that the $V_{\text{max}}$ of isoform A in phosphate buffer (60.89 ± 2.82 nmol of H$_2$NTP/min/mg of protein) is approximately twice that of isoforms B and C (29.07 ± 1.91 and 27.28 ± 0.86 nmol of H$_2$NTP/min/mg of protein, respectively). The only sequence differences among these three isoforms reside within the N-terminal extensions. The extension of isoform A is completely unique in sequence compared with the N-terminal domains of isoforms B and C. We hypothesized that specific residues or sub-domains within the N-terminal extensions of B and C act as negative regulators of GTPCH activity. To explore this possibility, various truncations of the N-terminal extension of recombinant isoform C were assayed for activity and compared with recombinant full-length isoforms and common region (Fig. 4). These experiments resulted in distinct and important observations. First, the common region alone (207 amino acid residues) exhibited significantly lower activity compared with the full-length isoforms; residues within the N-terminal extensions are therefore required for full enzymatic activity. Conversely, a truncation removing the initial 30 amino acid residues of isoform C had no significant effect on activity compared with full-length isoform C. This result suggests that amino acids 31–117 of the N-terminal extension of isoform C are required for full activity, whereas the first 30 amino acids are expendable in relation to activity.

Surprisingly, removal of the first 58 amino acid residues of isoform C resulted in a significant elevation in activity compared with full-length isoform C. In fact, the activity for the −58 isoform C truncation parallels that of full-length isoform A. This result suggests that a region of the N-terminal extension of isoform C, from amino acid residues 31 to 58, serves to negatively regulate the enzyme and is thus responsible for the lowered enzymatic activity of isoform C compared with isoform A. This negative regulatory region is shared between isoforms B and C, suggesting that this regulatory property is common to both isoforms.

Potential Phosphorylation Sites Reside within the Candidate Negative Regulatory Domain of **Drosophila** GTPCH Isoforms B and C—It has been suggested previously that mammalian GTPCH is regulated via phosphorylation and that phosphoryl-
isoform A, are phosphorylated (32, 33). These results suggest that full-length isoforms B and C serve as substrates for both PKA and PKC and that phosphorylation by these kinases occurs within the N-terminal extensions of these isoforms.

To establish whether Drosophila GTPCH can be phosphorylated by these kinases, we utilized \[^{32}P\]ATP with PKA (Fig. 5) and PKC (supplemental Fig. S4). Full-length isoforms B and C are phosphorylated (lanes 6 and 7) by both PKA and PKC, whereas the common region alone (lane 4) is not phosphorylated by either kinase. Full-length isoform A is not phosphorylated by PKA, and only a faint band is observed with PKC (lane 5). The N-terminal extensions of isoforms B and C, but not of isoform A, are phosphorylated (lanes 1–3). These results suggest that full-length isoforms B and C serve as substrates for both PKA and PKC and that phosphorylation by these kinases occurs within the N-terminal extensions of these isoforms.

To determine the functional significance of this post-translational modification of GTPCH isoforms B and C, recombinant isoforms were treated with PKA and subsequently assayed for enzymatic activity (Fig. 6). Both isoforms exhibit an elevation in activity following PKA treatment, indicating that Drosophila GTPCH isoforms B and C are activated by phosphorylation as suggested by earlier studies in mammalian cell culture (32, 33).

To explore the possibility that the serine-rich, candidate negative regulatory domain is regulated by phosphorylation, we modified Ser\(^{37}\) to glutamic acid to mimic phosphorylation at this site. The enzymatic activity of S37E is significantly higher than wild-type GTPCH isoform C (Fig. 7). This suggests that phosphorylation of residues within the candidate negative regulatory domain may serve to alleviate the negative regulation imposed on the enzyme by the native domain.

We hypothesized that substituting alanine for Ser\(^{37}\) would have no significant effect on enzymatic activity, because removal of the first 58 amino acids, including Ser\(^{37}\), results in higher activity as demonstrated by truncation analysis. Contrary to our expectations, the S37A substitution greatly reduced the activity of wild-type GTPCH isoform C (Fig. 7), suggesting that the candidate regulatory domain interacts with and plays a pivotal role at the active site of the enzyme. This regulatory interaction has not been reported previously for either GTPCH alone or GTPCH and GFRP.

**DISCUSSION**

Although it has been hypothesized that the N-terminal domain of GTPCH plays regulatory roles, we present here the first direct evidence of this phenomenon. Drosophila GTPCH is particularly amenable to this work, because the various alternative isoforms differ only in their N-terminal sequences, giving each isoform potentially distinct regulatory properties. The
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The serine residue at position 37 of the N-terminal extension of isoform C was mutated to alanine (S37A) and glutamic acid (S37E) and assayed for activity. S37A results in a significant decrease in activity compared with full-length C, whereas S37E results in a significant elevation in activity. Values are means ± S.E. from two independent experiments, each with triplicate determinations. **, p < 0.01; *** p < 0.001 (one-way ANOVA with Bonferroni post test).

Recrystallization of the aforementioned isoform C Ser37 to alanine drastically lowers the $V_{\text{max}}$ particularly because the complete removal of the negative regulatory region elevates the $V_{\text{max}}$ 2-fold. Such distinct effects of modulating a single amino acid within this regulatory region suggest that amino acids within the negative regulatory region may themselves be interacting at or near the active site of the full enzyme and thereby inhibiting the enzyme’s activity.

Phosphorylation of residues within the negative regulatory region may alter the conformation of the protein, thereby changing the state of this interaction and leading to an activated enzyme (Fig. 8B). This model for regulation of the enzyme by the negative regulatory region is supported by various lines of evidence, including the truncation and site-directed mutation analyses, as well as the demonstrated phosphorylation of the N-terminal extensions of isoforms B and C. Post-translational modification of mammalian GTPCH by PKC has been demonstrated previously, with the phosphorylated enzyme exhibiting elevated activity (32, 33). Likewise, we note a significant elevation in enzymatic activity for GTPCH isoforms B and C following treatment with PKA. Although we have not established that regulation by phosphorylation occurs in vivo,
our results parallel those obtained in the mammalian cell culture models. Ongoing studies of transgenic Drosophila and tissue extracts will address in vivo functions of phosphorylation.

The predicted site of phosphorylation by PKC for the mammalian enzymes lies within the core domain of the enzymes (Ser\textsuperscript{167}, mouse GTPCH), which is conserved in Drosophila (Ser\textsuperscript{249}, isoform C). However, we find that the N-terminal extensions of Drosophila isoforms B and C, and not the core catalytic region containing Ser\textsuperscript{249}, are phosphorylation targets for both PKC and PKA. Structure-function analyses will serve to further characterize this regulatory region and pinpoint the sites of phosphorylation within the N termini of the various isoforms. We note that Ser\textsuperscript{19} in human GTPCH, which lies in the N-terminal arm of that enzyme, is a predicted target for PKC. It will be interesting to determine whether Ser\textsuperscript{19} and Ser\textsuperscript{167} in the mammalian protein serve regulatory functions.

Taken together, these data suggest that the various N-termi-
nal arms of Drosophila GTPCH have evolved to serve multiple roles in regulating BH₄ levels. The isoform-specific regulatory properties, most divergent between isoform A and isoforms B and C, help to explain the different spatiotemporal expression patterns of these isoforms during development. The necessity for acute regulation of cellular concentrations of BH₄ cannot be overemphasized. The precise and rapid regulation of GTPCH is a primary requirement for the synthesis and activity of three key neurotransmitters: dopamine, serotonin, and nitric oxide. GTPCH is therefore expected to be a sensitive target for complex mechanisms that modulate each of these neurotransmitter systems.

Although Drosophila lacks the regulatory protein GFRP, the Drosophila enzyme alone is capable of a broad spectrum of the regulatory interactions exhibited or hypothesized for the mammalian enzyme. Thus, despite structural disparities between the Drosophila and mammalian regulatory components, the functional convergence suggests that Drosophila can accurately model organismal consequences of GTPCH mutations. In particular, the capacity to efficiently generate transgenic animals in the Drosophila system suggests that it will be possible to model the effects of genetic variation within regulatory domains and to examine, in vitro and in vivo, functional interactions with downstream target pathway components of dopamine, serotonin, and nitric oxide synthesis.

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REFERENCES

1. Nagatsu, T., Levitt, M., and Udenfriend, S. (1964) J. Biol. Chem. 139, 2910–2917
2. Lovenberg, W., Jequier, E., and Sjoerdsma, A. (1967) Science 155, 217–219
3. Kaufman, S., Pollock, R. J., Summer, G. K., Das, A. K., and Hajra, A. K. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 248–254
4. Tiets, A., Lindberg, M., and Kennedy, E. (1964) J. Biol. Chem. 239, 4081–4090
5. Kaufman, S., Pollock, R. J., Summar, G. K., Das, A. K., and Hajra, A. K. (1990) Biochim. Biophys. Acta 1040, 19–27
6. Tayeh, M. A., and Marletta, M. A. (1989) J. Biol. Chem. 264, 19654–19658
7. Kwon, N. S., Nathan, C. F., and Stuehr, D. J. (1989) J. Biol. Chem. 264, 20496–20501
8. Brown, G. M. (1985) in Folate and Pteridines (Blakely, R. J., and Benkovic, S. J., eds) pp. 115–154, John Wiley and Sons, New York
9. Ichinose, H., Ohye, T., Takahashi, E., Seki, N., Hori, T., Segawa, M., Nomura, Y., Endo, K., Tannaka, H., Tsujii, S., Fujita, K., and Nagatsu, T. (1994) Nat. Genet. 8, 236–242
10. Nagatsu, T., and Ichinose, H. (1997) J. Neurotransm. Suppl. 49, 203–209
11. Hirano, M., Yangtara, T., and Ueno, S. (1998) Am. J. Physiol. 275, H2165–H2170
12. Hwu, W. L., Yeh, H. Y., Fang, S. W., Chang, H. S., Chiu, Y. W., and Lee, Y. M. (1994) Biochem. Biophys. Res. Commun. 201, 212–215
13. Huck, W. L., Man, H., and Sjoerdsma, A. (1967) J. Biol. Chem. 242, 995–999
14. Chen, X., Ranganayakulu, G., and O'Donnell, J. M. (1997) Biochem. Biophys. Res. Commun. 239, 408–412
15. Hwu, W. L., Yeh, H. Y., Fang, S. W., Chang, H. S., Chiu, Y. W., and Lee, Y. M. (1994) Biochem. Biophys. Res. Commun. 201, 212–215
16. Hwu, W. L., Yeh, H. Y., Fang, S. W., Chang, H. S., Chiu, Y. W., and Lee, Y. M. (1994) Biochem. Biophys. Res. Commun. 201, 212–215
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and Bacher, A. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 13567–13572
51. Futahashi, R., and Fujiwara, H. (2006) Insect Biochem. Mol. Biol. 36, 63–70
52. Blom, N., Gammeltoft, S., and Brunak, S. (1999) J. Mol. Biol. 294, 1351–1362
53. Kapatos, G., and Hirayama, K. (1992) J. Neurochem. 59, 2048–2055
54. Kure, S., Hou, D., Ohura, T., Iwamoto, H., Suzuki, S., Sugiyama, N., Sakamoto, O., Fujii, K., Matsubara, Y., and Narisawa, K. (1999) J. Pediatr. 135, 375–378
55. Meininger, C. J., Cai, S., Parker, J. L., Channon, K. M., Kelly, K. A., Becker, E. J., Wood, M. K., Wade, L. A., and Wu, G. (2004) FASEB J. 18, 1900–1902
56. Krishnakumar, S., Burton, D., Rasco, J., Chen, X., and O’Donnell, J. (2000) J. Neurogenet. 14, 1–23