Differential Regulation of Drosophila Tyrosine Hydroxylase Isoforms by Dopamine Binding and cAMP-dependent Phosphorylation*

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Tyrosine hydroxylase (TH) catalyzes the first step in dopamine biosynthesis in Drosophila as in vertebrates. We have previously reported that tissue-specific alternative splicing of the TH primary transcript generates two distinct TH isoforms in Drosophila, DTH I and DTH II (Birman, S., Morgan, B., Anzivino, M., and Hirsh, J. (1994) J. Biol. Chem. 269, 26559–26567). Expression of DTH I is restricted to the central nervous system, whereas DTH II is expressed in non-nervous tissues like the epidermis. The two enzymes present a single structural difference; DTH II specifically contains a very acidic segment of 71 amino acids inserted in the regulatory domain. We show here that the enzymatic and regulatory properties of vertebrate TH are generally conserved in insect TH and that the isoform DTH II presents unique characteristics. The two DTH isoforms were expressed as apoenzymes in Escherichia coli and purified by fast protein liquid chromatography. The recombinant DTH isoforms are enzymatically active in the presence of ferrous iron and a tetrahydrobiopterin co-substrate. However, the two enzymes differ in many of their properties. DTH II has a lower $K_m$ value for the co-substrate (6R)-tetrahydrobiopterin and requires a lower level of ferrous iron than DTH I to be activated. The two isoforms also have a different pH profile. As for mammalian TH, enzymatic activity of the Drosophila enzymes is decreased by dopamine binding, and this effect is dependent on ferrous iron levels. However, DTH II appears comparatively less sensitive than DTH I to dopamine inhibition. The central nervous system isoform DTH I is activated through phosphorylation by cAMP-dependent protein kinase (PKA) in the absence of dopamine. In contrast, activation of DTH II by PKA is only manifest in the presence of dopamine. Site-directed mutagenesis of Ser^224, a serine residue occurring in a PKA site conserved in all known TH proteins, abolishes phosphorylation of both isoforms and activation by PKA. We propose that tissue-specific alternative splicing of TH has a functional role for differential regulation of dopamine biosynthesis in the nervous and non-nervous tissues of insects.

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Tyrosine hydroxylase (TH) (tyrosine 3-monooxygenase, EC 1.14.16.2) is an eukaryotic enzyme catalyzing the first and rate-limiting step in dopamine and other catecholamine biosynthesis, i.e. the hydroxylation of the monophenol amino acid $l$-tyrosine to produce the ortho-diphenol $l$-dihydroxyphenylalanine (2, 3). The enzyme is active in the presence of ferrous iron, O$_2$, and a tetrahydrobiopterin co-substrate. A single gene encodes TH, which is required for embryonic development and survival in mammals (4, 5). In vertebrates, TH activity is exquisitely regulated at each step of its expression: control of gene transcription, RNA alternative processing, mRNA stability, and direct modulation of the enzyme by catecholamine feedback inhibition and protein kinase activation (6–8).

In contrast, much less is known on the regulatory properties of tyrosine hydroxylase in insects. Mutations in the Drosophila pale locus, which corresponds to DTH (9–11), result in unpigmented embryos that are unable to hatch. It has been shown that dopamine has a dual function in insects, acting as a neurotransmitter in the central nervous system (12, 13) and as a precursor molecule required for pigmentation and hardening of the cuticle (14–16). The TH enzyme is composed of a carboxyl-terminal catalytic domain and an amino-terminal regulatory domain (17–21). The catalytic domain has been well conserved in Drosophila TH (22). The regulatory domain is not conserved but contains a potential protein kinase A (PKA) site occurring at Ser^224 that is homologous to Ser^228, the major site of phosphorylation by PKA in vertebrate TH (8). Two different forms of TH proteins have been found in Drosophila melanogaster, which are produced through alternative splicing of a single copy gene (1). The major form, Drosophila TH Type I (DTH II), contains a very acidic segment of 71 amino acids inserted in the regulatory domain close to the PKA phosphorylation site. The two DTH isoforms are expressed in distinct tissues; DTH I is specific of the nervous tissue, whereas DTH II is widely expressed in non-nervous tissues (1). DTH II is strongly expressed in the epidermis, or hypoderm, the single-layered epithelium that covers the insect body and secretes the cuticle.

To compare the kinetic and regulatory properties of the two Drosophila TH isoforms in vitro, we have expressed each of these molecules as recombinant apoenzymes in a bacterial expression system. Both isoforms were produced at a high level and purified. We show here that the two enzymes differ in many of their properties, including their pH profiles, iron requirements, and $K_m$ values for (6R)-5,6,7,8-tetrahydrobiopterin (BH$_4$). In addition, the two enzymes are differentially regulated

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* The abbreviations used are: TH, tyrosine hydroxylase; BH$_4$, (6R)-5,6,7,8-tetrahydrobiopterin; DTH, Drosophila tyrosine hydroxylase; HTH, human tyrosine hydroxylase; PCR, polymerase chain reaction; PKA, cAMP-dependent protein kinase; Mes, 2-(N-morpholino)ethane-sulfonic acid; bp, base pairs; PAGE, polyacrylamide gel electrophoresis.
by dopamine feedback inhibition and activation by cAMP-depen-
dent phosphorylation. Our data suggest that the acidic extension in the regulatory domain of DTH II endogenously activates the enzyme. The structural difference between the two DTH isoforms could therefore have a functional role and correspond to a differential regulation of dopamine biosynthe-
sis in nervous and non-nervous tissues of insects.

**EXPERIMENTAL PROCEDURES**

*Construction of the DTH Expression Vectors*—The cDNA clones pDTHcDNA1 and pDTHcDNA2, which encode DTH Type I and Type II, respectively, were isolated previously. cDNA segments containing the complete coding sequence of each DTH isoform were subcloned into the Escherichia coli expression vector pET-11a (23). First, a NdeI site was introduced at the translation start site of both cDNAs by site-
directed mutagenesis. The method we used was a modification of the three primer PCR mutagenesis procedure. PCRs were carried out with Vent polymerase (New England Biolabs) in a 50-
μl final volume as described by Marini et al. (24) using 1 ng of the plasmid pDTHDNA as a template. The primary reaction was performed with a 5’ external sense DTH oligonucleotide primer (OTH1, 5’-TTGGCCCTAAAGACTTG TGGC) and an internal mutagenesis antisense DTH primer with two mismatches (OTH1, 5’-CCGGCATCATGGTTTGTGTG; mis-
mated site underlined) and a NdeI site introduced at the translation start site (25). The product of this PCR, a double-stranded 442-bp DNA segment intermediate in the modified polymerase chain reaction (PCR), was used as a “megaprimer” in a second similar PCR with a 3’ external antisense DTH primer (OTH2, 5’-CAACAATATCTGTC TCATGGGACAGC). The final 645-bp amplification product was di-
gested with XbaI and XhoI and inserted to replace the corresponding sequence in pDTHcDNA1 and pDTHcDNA2. Finally, NdeI-
BamHI cDNA segments containing the coding sequence of the DTH isoforms Type I and Type II were ligated to the vector pET-11a previously digested with NdeI and BamHI to generate the recombinant expression vectors pEDTHI and pEDTHII, respectively.

To replace Ser28 with Arg in both DTH isoforms, a mutagenesis DTH sense primer was synthesized (OTHm2, 5’-CTGGCGCCGCGCTGGTCTTGG-3’). A double-stranded 131-bp DNA segment intermediate was amplified from pDTHcDNA1 with the primers OTH2 and OTHm2. This mutated 131-bp segment and the 442-bp segment obtained previously with the primers OTH1’ and OTHm1 were joined by a second PCR. The final doubly mutated 645-bp amplification product was used as de-
scribed for the previous constructions to generate the recombinant expression vectors pEDTHII(32R) and pEDTHII(32R). All mutations were checked by PCR and confirmed by double-stranded DNA sequencing.

*Expression of Recombinant DTH Isoforms*—DTH expression vectors were introduced by electroporation into E. coli BL21(DE3) cells (Novagen), which do not express the lon and ompT proteases (23). Fresh cultures inoculated with a 1:100 dilution of a 10–11 h preculture were grown in M9ZB (23) plus 100 μg/ml carbenicillin for 3.5 h at 37 °C. Cells were then diluted and resuspended in fresh medium. A 1-liter culture in M9ZB plus 100 μg/ml carbenicillin was inoculated with 10 ml of these cells and grown for 2 h at 37 °C with vigorous shaking until the A600 was equal to 0.4–0.5. To minimize the formation of inclusion bodies, the culture was then transferred at 18 °C with gentle shaking (125 rpm). DTH expression was induced 30 min later by adding isopropyl β-D-thiogalactopyranoside to 1 mM. After overnight (13–14 h) incubation at 18 °C, cells were harvested by centrifugation at 5,000 × g for 15 min. Weighted pellets (5.9–6.2 g) were washed once in ice-cold 0.3 M sucrose, 0.1 M EDTA, and 50 mM Tris-HCl, pH 7.5. Cells were then resuspended at 0.15–0.20 g/ml in the same ice-cold buffer supplemented with 0.5 mM dithiothreitol, 1 μM leupeptin, 0.5 mM pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml lysozyme, and 0.1 mg/ml DNase. Cell lysis was completed by sonication or by using a French press and checked by microscope examination. Insoluble proteins were removed by centrifugation at 27,000 × g for 60 min at 4 °C, and the clear supernatant (bacterial soluble extract) was complemented with glycerol to 10% (v/v) and either stored at −80 °C or used immediately for DTH purification.

*Determination of TH Enzymatic Activity*—TH enzymatic activity was assayed by measuring the enzymatic release of tritium from L-[3,5-3H]tyrosine. For kinetic studies, the standard conditions for the assay were 50 mM K-Hepes, 150 μM l-tyrosine, 2.5 μM CI-mannitol, 3.5-Htyro-
sine (50 CI/ml, Amersham), 20 μM BH4, 10 μM ferrous ammonium sulfate, 15 μM β-mercaptoethanol, 0.07 mg/ml catalase (Sigma), with 0.2% (w/v) BSA in a purified DTH isoform in a volume of 1 ml after a 2–3 min equilibration of the mixture at the assay temperature (25 or 29 °C), the reaction was started by the addition of BH4. Assays were stopped by the addition of 1 ml of 7.5% activated carbon (Darco G60, Fluka) suspension in 1 N HCl as described by Reinhard et al. (26). The charcoal was sedimented, and an aliquot (100 μl) of the supernatant containing the tritiated water was mixed with 4 ml of Ready Safe scintillant (Beckman). Controls were obtained without BH4. To stay under initial velocity conditions, reactions were quenched after 2 min, and the amount of enzyme assayed was kept below 0.5 unit. One unit of enzyme produces 1 μmol of ortho-diphenol l-dihydroxyphenylalanine/ min at 25 °C. Under these conditions, assays were linear with time and with the amount of enzyme. Linearity was not conserved with larger amounts of enzyme and longer reaction times (15 min) probably because of O2 consumption. Stocks of ferrous ammonium sulfate were stored in aliquots at −20 °C and not reused after thawing (27). Michaelis-Menten constants were determined from Lineweaver-Burk curves analyzed by the least squares curve fitting method with the MacCurveFit software (Kevin Raner). To determine the pH dependence of DTH activity, the assay buffer K-Hepes was replaced by a constant ionic strength buffer (50 mM sodium acetate, 50 mM Mes, 100 mM Tris-HCl) (28, 29). To determine the effect of dopamine on enzyme activity, DTH was pre-
incubated with dopamine for 5 min at 25 °C in the assay mixture before the reaction was started. Most experiments were conducted in parallel for the two isoforms to compare DTH activity and regulation in closely similar conditions. All data are the mean of duplicate or triplicate determinations.

*Phosphorylation of DTH Isoforms*—Recombinant DTH isoforms were phosphorylated by the catalytic subunit of PKA. Either the purified isoforms or the soluble extracts from DTH-expressing E. coli cells were used. The conditions of phosphorylation were 5–15 min at 30 °C in 25 mM Hepes, pH 7.0, 10 mM MgCl2, 2 mM ATP, 2.5 mM spermidine, 0.5 mM EDTA, 0.5 mM EGTA, 0.025 units/ml PKA catalytic subunit (New England Biolabs) in a volume of 25 μl. Nonphosphorylated controls were
The nature of the recombinant proteins synthesized in E. coli was further checked on Western blots probed with an affinity-purified antibody to rat TH and an antibody raised to the specific acidic domain of DTH II. Fig. 2B (left panel) shows that both induced peptides are recognized by the antibody to rat TH, confirming that these molecules are TH proteins. In addition, the antibody to DTH II recognizes, as expected, the larger protein only (Fig. 2B, right panel). A band migrating at the same apparent molecular weight was detected with this antibody on a Western blot of proteins extracted from Drosophila heads (not shown), demonstrating that the native and recombinant DTH II proteins migrate identically on SDS-PAGE.

We found that DTH expression has to be induced at a low temperature (18 °C) to recover soluble and active proteins. For both isoforms, a tyrosine hydroxylase enzymatic activity was detected in the bacterial soluble extract from the induced cells, which was strictly dependent on the presence of iron and a reduced pteridine co-substrate in the assay mixture. No TH activity was found in control cells transformed with the nonrecombinant vector pET-11a.

Purification of DTH isoforms was carried out by ion exchange fractionation and ammonium sulfate precipitation followed by Mono-Q fractionation. The final fractions obtained were considerably enriched (Fig. 2C), although the enzymes were not purified to homogeneity. We estimated that TH activity was enriched approximately 11-fold for DTH I and 15-fold for DTH II in the purified fractions as compared with the bacterial expression system has been used previously to express and purify the DTH isoforms.

Enzymatic and regulatory properties of both DTH enzymes were analyzed in controlled conditions after expression in E. coli BL21(DE3) cells (23) and purification. The prokaryotic expression system has been used previously to express and characterize several TH isoforms from human and rat (21, 30–33). As an advantage, the TH protein produced in bacteria is essentially an unphosphorylated apoenzyme that contains a very low amount of bound iron (34) and no catecholamines. It is thus best suited to study the effect of phosphorylation and modulators on enzymatic activity.

In cells transformed with the recombinant expression vectors pEDTHI and pEDTHII (see “Experimental Procedures”), the addition of isopropyl β-D-thiogalactopyranoside induced an efficient biosynthesis of DTH I and DTH II proteins, which migrated with an apparent molecular mass of 58 and 79 kDa, respectively, on SDS-PAGE (Fig. 2A). For DTH I, this estimation is in agreement with the molecular mass predicted from the coding sequence of the cDNA (57,862 Da). In contrast, the apparent molecular mass of DTH II was higher than expected, because the sequence predicts a protein of 65,996 Da. A minor band at 62 kDa is also detected specifically in the DTH II-expressing cells after induction; it is probably a degradation product. Treatment with a high salt or alkaline pH before electrophoresis did not modify the migration of DTH II (not shown), suggesting that this aberrant migration is not because of protein interactions. Such an anomalous mobility in SDS gels is not unusual with very hydrophilic proteins. The acidic segment in the regulatory domain of DTH II may prevent regular binding of SDS molecules and thus delay migration of the protein.

The results of various protein interactions were consistent with the conclusions reached on the basis of sequence comparisons. The N-terminal region of DTH II contains a specific acidic segment that may prevent interaction with other proteins. Such acidic segments have been observed in other enzymes and have been shown to be involved in protein-protein interactions.

Regulation of Drosophila Tyrosine Hydroxylase Isoforms—The nature of the recombinant proteins synthesized in E. coli was further checked on Western blots probed with an affinity-
Procedures) in which the rate of tyrosine hydroxylation is linear with time and enzyme quantity (Fig. 3). In these conditions, the specific activity of purified DTH II was found to be about twice the specific activity of purified DTH I (Table I). The Michaelis constants of the DTH enzymes for L-tyrosine and the co-substrate BH4 are presented in Table II. The two isoforms differ in their $K_m$ value for BH$_4$, which was found to be reproducibly 1.5-fold lower for DTH II. BH$_4$ has an inhibitory effect on DTH I and II activities but at higher levels (400 μM and above) (not shown). The kinetic data obtained for the recombinant Drosophila enzymes are comparable to the values obtained for vertebrate TH expressed in E. coli (32, 33).

Another significant difference found between the two DTH enzymes is their pH activity profile (Fig. 4). Activity of DTH I is maximal at pH 7 and much reduced at acidic and alkaline pH. Such a bell-shaped pH profile was reported for recombinant rat TH (35). In contrast, DTH II has a broader profile, and its activity is not markedly reduced in alkaline conditions (Fig. 4).

Effect of Ferrous Iron, Dopamine, and Heparin on DTH Activity—Vertebrate tyrosine hydroxylase is a metalloprotein that requires ferrous iron for enzymatic activity (27, 34, 36). The iron requirement of purified DTH I and II has been compared by varying ferrous ion concentration in the assay mixture. We found that iron is necessary for DTH enzymatic activation, but the iron dependence profile is different for the two isoforms (Fig. 5). These results were confirmed with two different enzyme preparations of each isoform. The approximate iron concentration required half-maximal activation of DTH I and II is 10 and 2 μM, respectively. Interestingly, DTH I activity was not detectable at 2 μM iron, whereas a slight activity could be detected for DTH II even with no iron added in the assay mixture (Fig. 5). Activity was decreased at 167 μM iron to 71 and 93% of the maximal value for DTH I and DTH II, respectively (not shown).

Dopamine, one of the end products of the catecholamine biosynthesis pathway, has been shown to inhibit vertebrate TH activity (37–40). We found that this regulation also occurs in Drosophila, although DTH seems to be less sensitive to dopamine inactivation than vertebrate TH. Activity of both DTH isoforms is inhibited in the presence of 20 μM dopamine, and this effect is dependent on ferrous iron concentration, the inhibition being more pronounced at higher iron level. DTH II appears more resistant to dopamine feedback inhibition than DTH I (Fig. 6). In addition, lower amounts of iron are required to activate DTH II than to allow for dopamine inhibition of its activity. Thus, at 10 μM ferrous iron, a value that could be close to the physiological iron concentration in cells, DTH II is both fully activated (Fig. 5) and resistant to dopamine inhibition (Fig. 6).

It is known that TH activity is stimulated by the binding of polyanions like heparin (41–43). The mammalian TH protein contains a critical sequence for heparin binding in the regulatory domain that has been mapped to amino acids 68–90 (44). However, this sequence is not conserved in Drosophila TH, and we have found that 1 mg/ml heparin in the assay mixture has no significant effect on DTH activity (not shown).

Effect of Phosphorylation by PKA on DTH and S32R Mutant Activity—One of the major types of regulation of vertebrate TH is through activation by cAMP-dependent phosphorylation of a conserved serine residue located in the regulatory domain of the molecule (Ser40 in rat TH) (6–8). This PKA site has been well conserved in Drosophila TH occurring at Ser32 (Figs. 1 and 7A). Phosphorylation of the DTH isoforms by the catalytic subunit of PKA was carried out in the presence of radioactive ATP. Autoradiography of the phosphorylation product shows that DTH I and II are both rapidly phosphorylated (Fig. 7C, lanes 1 and 3). To check that the enzymes were phosphorylated at the serine residue occurring in the conserved PKA site, Ser32 was mutated to an arginine by site-directed mutagenesis (Fig. 7B). The mutant isoforms thus obtained, DTH I (S32R) and DTH II (S32R), were expressed in E. coli and found to be enzymatically active. As shown in Fig. 7C, lanes 2 and 4, phosphorylation by PKA is abolished in these S32R mutants. Fig. 8A shows that phosphorylation of DTH I by the catalytic subunit of PKA in the bacterial soluble extract leads to a significant increase in TH activity (327 ± 21%, mean of four independent experiments). The activity of purified DTH I is also significantly stimulated by phosphorylation with PKA (175 ± 20%, mean of three independent experiments). The activity of the mutant enzyme DTH I (S32R) is not increased by the catalytic subunit of PKA (Fig. 8B), demonstrating that stimulation of DTH I by PKA directly results from the phosphorylation of Ser32.

In contrast to DTH I, we found that activity of the DTH II isoform is not stimulated by PKA either in the bacterial soluble extract (Fig. 8C) or after purification (shown in Fig. 9). As expected, the mutant enzyme DTH II (S32R) is not regulated by PKA as well (Fig. 8D). However, phosphorylation of DTH II by PKA activates the enzyme in the presence of dopamine and a high iron concentration, bringing back the enzyme activity close to the level observed in the absence of dopamine (Fig. 9). The phosphorylated enzyme even appears slightly more active in the presence of dopamine (Fig. 9), probably because dopamine binding stabilizes TH (39). Thus, phosphorylation by PKA has no effect on basal DTH II activity and only activates efficiently the enzyme inhibited by dopamine.

**DISCUSSION**

Alternative splicing of the TH primary transcript generates two different TH isoforms in Drosophila. The major isoform, DTH II, contains a specific 71-amino acid hydrophilic segment in the amino-terminal regulatory domain (Fig. 1). Messenger RNA localization showed that DTH I is only expressed in the central nervous system, and DTH II is expressed at a high level in the epidermis (1). In addition, no DTH II protein can be detected in the Drosophila central nervous system by immunolabeling with a polyclonal antibody to the specific hydrophilic segment of DTH II. Alternative splicing of Drosophila TH is then strictly tissue-specific. To address the physiological relevance of these observations, it was interesting to compare the enzymatic and regulatory properties of the DTH isoforms. Because of the low level of TH activity in Drosophila, it would have been very difficult to extract and purify the TH enzymes from insect tissues. Therefore, the two isoforms were analyzed after high level expression in E. coli and purification. In addition, because catecholamine biosynthesis or cAMP-dependent phosphorylation do not occur in bacteria, the prokaryotic expression system is well adapted to study TH regulation.

**TABLE I**

| Enzyme          | Fraction | Total protein | Total activity | Specific activity |
|-----------------|----------|---------------|----------------|------------------|
| DTH I           | Cell lysate | 440           | 6,865          | 15.6             |
|                 | Mono-Q   | 9.6           | 1,586          | 165.2            |
| DTH II          | Cell lysate | 452           | 11,566         | 25.6             |
|                 | Mono-Q   | 7.5           | 2,882          | 384.3            |

* TH activity was determined in standard conditions at 25 °C.

* From 6 g of cells.
Regulation of Drosophila Tyrosine Hydroxylase Isoforms

The Drosophila TH isoforms expressed in E. coli were found to be enzymatically active. In addition, they are bound by TH-specific antibodies (Fig. 2). The anomalous migration of DTH II on SDS-PAGE indicates that the additional hydrophilic segment may alter the structure of the protein or prevent regular binding of SDS molecules. Although purified DTH II seems to have a higher specific activity than purified DTH I, the enzymes were probably not pure enough to allow for precise determination of their catalytic constants. In addition, our results show that activity of the DTH enzymes dramatically depends on assay conditions.

The $K_m$ for tyrosine was found to be comparable for the two DTH isoforms. In contrast, the $K_m$ value for BH$_4$ is higher for DTH II (Table II). Thus, the presence of an additional acidic amino acid segment in the regulatory domain of DTH II decreases the $K_m$ for BH$_4$ as compared with DTH I. Interestingly, it has been reported that phosphorylation of recombinant rat TH by PKA decreases the $K_m$ value for BH$_4$ (32) (see below).

$\text{TH}$ is a metalloprotein that requires ferrous iron for activity (27, 34, 36). A site-directed mutagenesis study (45) and crystal structure of the catalytic domain of rat TH (46) have shown that the iron atom is localized within the active site cleft of the enzyme and is coordinated by three amino acid residues: His-331, His-336, and Glu-376. These amino acids are conserved in

\begin{table}[h]
\centering
\begin{tabular}{lcc}
\hline
 & $K_m$ & $Tyr^a$ & $BH_4^a$
\hline
DTH I & 22.7$^a$ & 48.5$^a$ & 11.8$^a$
DTH II & 14.8$^a$ & 56.4$^a$ & 7.4$^a$
\hline
\end{tabular}
\caption{Apparent Michaelis constants of recombinant Drosophila TH isoforms. Results represent the mean ± S.E. of four independent determinations under standard conditions (pH 7 and 25°C).}
\end{table}

The enzymatic activity of purified DTH isoforms was determined in the presence or absence (control) of 20 $\mu$M dopamine with various levels of ferrous ammonium sulfate (Fe (II)) added in the assay reaction mixture. Results are the mean of three independent experiments carried out with two different preparations of each enzyme. Activity is expressed as percent of the mean value determined at 55 $\mu$M ferrous iron for each DTH isoform.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig5.png}
\caption{Ferrous iron requirement for the enzymatic activity of DTH I and II. Enzymatic activity of purified DTH I (open circles) and DTH II (closed circles) was determined in standard conditions with various levels of ferrous ammonium sulfate (Fe (II)) added in the assay reaction mixture. Results are the mean of three independent experiments. The ferrous iron concentration (Fe (II)) is plotted in logarithmic scale. The asterisks denote iron concentrations for which the effect of dopamine is significantly different on the two DTH isoforms as determined by the independent Student's $t$ test ($p < 0.03$).}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig6.png}
\caption{Inhibition of DTH activity by dopamine. Enzymatic activity of purified DTH isoforms was determined in the presence or absence (control) of 20 $\mu$M dopamine with various levels of ferrous iron in the assay mixture (6.2, 18.5, 55, and 167 $\mu$M). Activity is expressed as percent of the control without dopamine. Open circles, DTH I; closed circles, DTH II. Each determination is the mean of four to six independent experiments. The ferrous iron concentration (Fe (II)) is plotted in logarithmic scale. The asterisks denote iron concentrations for which the effect of dopamine is significantly different on the two DTH isoforms as determined by the independent Student's $t$ test ($p < 0.03$).}
\end{figure}
the Drosophila enzymes, corresponding to His-338, His-343, and Glu-383, respectively, in DTH I. Native TH purified from vertebrate tissues contain bound catecholamines, and catecholamine binding prevents the release of the bound iron (47–49). Because E. coli contains no catecholamines, iron may be less strongly bound to TH enzymes expressed in bacteria than to native TH enzymes extracted from tissues. When expressed in E. coli and purified, the human TH isoforms are indeed recovered as practically iron-free apoenzymes (32, 34). In addition, the purification buffer we used contains dithiothreitol, which has been shown to increase the rate of iron release from TH (34). Consequently, the purified DTH isoforms can be similarly considered as apoenzymes practically devoid of iron.

Although the two Drosophila TH enzymes have been purified by the same procedure, our data show that DTH I and DTH II require different levels of iron to be activated (Fig. 5). Very low levels of iron are sufficient to ensure full activation of DTH II. The following two hypotheses can be proposed to interpret this observation: either the iron binding site is less accessible in DTH I than in DTH II or the regulatory acidic domain in DTH II increases enzyme affinity for iron. Consequently, we could predict that the central nervous system enzyme DTH I would be more sensitive than DTH II to cellular variations in the level of iron. Could iron be an endogenous regulator of TH activity? Free iron in the cytosol is kept at a low level (probably below 10 μM) by incorporation into protein chelators like ferritin (50). Human brain TH extracted from the caudate nucleus is activated more than 10-fold by incubation with ferrous ion (51), and Haavik et al. (34) have proposed that a significant fraction of human TH is not saturated with iron in vivo. In addition, these authors have reported that recombinant HTH1 requires a lower level of iron (7.8 μM) than HTH2 (13.5 μM) for half-maximal activation. These results and ours suggest that it would be interesting to compare the iron activity profile for the different vertebrate TH isoforms.

Dopamine and other catecholamines inhibit and stabilize vertebrate TH activity (37–40), and iron stimulates dopamine binding (31). We have shown here that this regulation is conserved in Drosophila TH and that the level of inhibition by dopamine depends on ferrous ion concentration. For vertebrate TH, it has been demonstrated that catecholamine binds to the active site iron oxidized in a ferric redox state (47, 49), thus stabilizing the enzyme in an inactivated form (32). Phosphorylation by PKA decreases the binding affinity of human TH for dopamine (40, 52), and conformation studies suggest that: 1) there is a direct interaction between the region surrounding the PKA site in the regulatory domain, and the iron binding site occurring close to the active site in the catalytic domain; 2) this interaction is required for dopamine binding and inhibition of TH activity; and 3) this interaction is relieved by phosphorylation of Ser40. Interestingly, DTH II seems to be more resistant than DTH I to dopamine inhibition (Fig. 6),
sugestng that this isoform is at least partially in an activated state comparable to that of phosphorylated TH.

Another major difference we observed between DTH I and DTH II is the effect of phosphorylation. Both TH isoforms are good substrates for the PKA catalytic subunit (Fig. 7). Site-directed mutagenesis of Ser32 to an arginine abolished phosphorylation, demonstrating that only Ser32 can be phosphorylated by PKA in Drosophila TH. Similar results have been obtained in previous studies by site-directed mutagenesis of the homologous Ser40 in rat TH (32, 55). We have found that phosphorylation by PKA markedly stimulates DTH I activity in the absence of catecholamine. For recombinant rat TH expressed in E. coli, it has been shown that phosphorylation by PKA in the absence of dopamine results in a moderate decrease in the $K_m$ for BH$_4$ and no increase in $V_{max}$ (32). Such a decrease in $K_m$ was shown to induce only an apparent 2-fold activation of the human isoform HTH1 when assayed in standard initial velocity conditions (33). Therefore, a decrease in $K_m$ is probably sufficient to explain the strong activation of DTH I by PKA phosphorylation we observed in the bacterial extract in the absence of dopamine (Fig. 8). We have preliminary data suggesting that phosphorylation of DTH I by PKA increases the $V_{max}$ for BH$_4$ in these conditions (not shown), as is the case for recombinant rat TH in the presence of dopamine (32). Further work is needed to clarify this apparent discrepancy between mammalian TH and Drosophila TH I.

In contrast to DTH I, phosphorylation by PKA does not regulate DTH II in the absence of dopamine. It has been shown that phosphorylation of HTH1 by PKA induces a structural transition in the enzyme (53). Because DTH II is rapidly phosphorylated by PKA (Fig. 7), two hypotheses could be proposed for the lack of effect of phosphorylation on DTH II activity in the absence of dopamine. Either the amino acid segment inserted in the regulatory region prevents a conformational change required for enzyme activation or alternatively the TH enzyme containing this segment spontaneously presents a conformation similar to that of the phosphorylated enzyme. A comparable lack of regulation by PKA has been recently reported for the human isoform HTH3 in the absence of dopamine (33). On the opposite side, we show that phosphorylation of DTH II by PKA activates the enzyme in the presence of dopamine (Fig. 9). This result is in agreement with the proposal that the major mode of activation of TH by phosphorylation is the alleviation of catecholamine inhibition (31, 32, 56). We propose that the structure of the regulatory domain in DTH II precludes activation by phosphorylation in the absence of dopamine because the enzyme is already in a partially activated conformation, but phosphorylation can still reactivate the enzyme inhibited by dopamine. In the insect epidermis where DTH II is expressed, the unique properties of this isoform could be an advantage because a large amount of catecholamines has to be produced in a short period of time when a new cuticle is being created. For example, it is known that regulation of mammalian TH changes the pH dependence of the enzyme; activation by cAMP-dependent phosphorylation broadens the pH profile and shifts the pH optimum toward the basic side (8, 57), and inhibition by dopamine shifts the pH activity profile toward the acidic side (31, 54). Clearly, the pH dependence of DTH II resembles the profile of an activated TH enzyme. In addition, steady-state intracellular pH is 7.4–7.6 in Drosophila (58, 59). This slightly alkaline value is even increased in the presence of 20-hydroxyecdysone (+ 0.3 units) (59), a hormone that plays an important role in the larval moulting process. Therefore, the pH activity profile of DTH II could be an advantage for rapid dopamine biosynthesis in the epidermis during moulting.

In conclusion, we have shown in this study that a structural difference in the regulatory domain of Drosophila TH isoforms affects several properties of the enzymes, such as the $K_m$ for BH$_4$, pH dependence, ferrous iron activation, regulation by dopamine, and cAMP-dependent phosphorylation. Several lines of evidence indicate that the acidic segment in the regulatory domain of DTH II stabilizes the enzyme in an activated conformation. Conservation of a functional Ser$_40$ PKA site in all the TH sequences through evolution argues that this site is an essential feature of the enzyme. In vivo genetic experiments are now in progress to characterize further the role of the two DTH isoforms and the function of this PKA site in tissuespecific regulation of dopamine biosynthesis in Drosophila.

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