BJ www.biochemj.org

BJ Gene

Biochem. J. (2011) **434**, 133–141 (Printed in Great Britain) doi:10.1042/BJ20101561

online data

Divergence in enzyme regulation between *Caenorhabditis elegans* and human tyrosine hydroxylase, the key enzyme in the synthesis of dopamine

Ana C. CALVO*[1], Angel L. PEY†, Antonio MIRANDA-VIZUETE‡§, Anne P. DØSKELAND* and Aurora MARTINEZ*[1]

*Department of Biomedicine, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway, †Departamento de Química Física, Facultad de Ciencias, Universidad de Granada, 18071-Granada, Spain, ‡Centro Andaluz de Biología del Desarrollo (CABD-CSIC), Departamento de Fisiología, Anatomía y Biología Celular, Universidad Pablo de Olavide, E-41013 Sevilla, Spain, and §Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, E-41013 Sevilla, Spain

TH (tyrosine hydroxylase) is the rate-limiting enzyme in the synthesis of catecholamines. The *cat-2* gene of the nematode *Caenorhabditis elegans* is expressed in mechanosensory dopaminergic neurons and has been proposed to encode a putative TH. In the present paper, we report the cloning of *C. elegans* full-length *cat-2* cDNA and a detailed biochemical characterization of the encoded CAT-2 protein. Similar to other THs, *C. elegans* CAT-2 is composed of an N-terminal regulatory domain followed by a catalytic domain and a C-terminal oligomerization domain and shows high substrate specificity for L-tyrosine. Like hTH (human TH), CAT-2 is tetrameric and is phosphorylated at Ser^{35} (equivalent to Ser^{40} in hTH) by PKA (cAMP-dependent protein kinase). However, CAT-2 is devoid of characteristic regulatory mechanisms present in hTH, such as negative co-operativity for the cofactor, substrate inhibition or feedback inhibition exerted by catecholamines, end-products of the pathway. Thus TH activity in *C. elegans* displays a weaker regulation in comparison with the human orthologue, resembling a constitutively active enzyme. Overall, our data suggest that the intricate regulation characteristic of mammalian TH might have evolved from more simple models to adjust to the increasing complexity of the higher eukaryotes neuroendocrine systems.

Key words: *Caenorhabditis elegans*, dopamine, nervous system, phosphorylation, tyrosine hydroxylase.

INTRODUCTION

TH (tyrosine hydroxylase) belongs to the family of AAAHs (aromatic amino acid hydroxylases) together with PAH (phenylalanine hydroxylase) and the TPH (tryptophan hydroxylase) 1 and 2 [1,2]. The AAAHs are catalytically and structurally related, and they all require the cofactor BH_4 (tetrahydrobiopterin), ferrous iron and O_2 for catalysis. These enzymes have been reported to be mainly tetramers, organized in a three-domain arrangement, with a N-terminal regulatory domain that is the most divergent in sequence, a catalytic domain that is the most conserved among all the hydroxylases and a C-terminal oligomerization domain, consisting of dimerization and tetramerization motifs [3]. The only exceptions to this organization are the bacterial PAHs, which only have the catalytic domain [4,5].

In humans, only one gene encodes TH, in contrast with other organisms such as the red junglefowl *Gallus gallus*, the protozoan *Toxoplasma gondii*, or several fish species, such as *Danio rerio* (zebrafish), which have been reported to possess two genes encoding TH [6]. Despite the presence of a unique gene, the diversity in TH isoforms is important in humans, as four different transcripts are produced by alternative splicing [7]. Moreover, additional isoforms for hTH (human TH) have been described in pathological situations [8,9].

TH catalyses the hydroxylation of L-tyrosine into L-dopa, the rate-limiting step in the synthesis of catecholamines, important hormones and neurotransmitters in the neuroendocrine system of both vertebrates and invertebrates. Catecholamines have been implicated in multiple functions such as locomotion, modulation of some behavioural states, aging, learning and development [10,11]. Owing to the important functions of catecholamines, TH is a tightly regulated enzyme at different levels, such as gene expression, mRNA degradation and enzyme regulation by the cofactor, substrate, end-products and phosphorylation [12]. Among the most studied and conserved regulatory mechanisms in TH are feedback inhibition by catecholamines and activation of TH activity by phosphorylation, which operate in synergy. Thus phosphorylation of mammalian TH by PKA (cAMP-dependent protein kinase) at Ser^{40} increases the rate of catecholamine dissociation, resulting in a more active enzyme [13,14]. Three other phosphorylation sites at N-terminal threonine or serine residues have been described in mammalian TH [15], regulating enzymatic activity, stability or interaction with partners and membranes [16,17], although the physiological consequences are in fact not completely understood.

Phosphorylation by PKA appears to be a well-conserved regulatory mechanism and is present at different levels in the TH evolutionary history. *Drosophila melanogaster* TH has been described to be phosphorylated in both isoforms, even though the consequences of the phosphorylation are not identical [18]. *Manduca sexta*, the tobacco hornworm, also has a putative recognition sequence for phosphorylation by PKA (motif RRXS) in its TH sequence, although the occurrence of the phosphorylation has not been proven experimentally [19]. *T. gondii*, the only protozoan described so far with a TH in

Abbreviations used: AAAH, aromatic amino acid hydroxylase; BH_4, tetrahydrobiopterin; CAT-2-P, Ser^{35}-phosphorylated CAT-2; DTT, dithiothreitol; EST, expressed sequence tag; GST, glutathione transferase; hTH, human tyrosine hydroxylase; LC, liquid chromatography; MS/MS, tandem MS; ORF, open reading frame; PAH, phenylalanine hydroxylase; PKA, cAMP-dependent protein kinase; QTOF, quadrupole time-of-flight; RT, reverse transcription; SL, *trans*-splicing signal; TFA, trifluoroacetic acid; TH, tyrosine hydroxylase; TPH, tryptophan hydroxylase; UTR, untranslated region.

[1] Correspondence may be addressed to either of these authors (email ana.calvo@biomed.uib.no or aurora.martinez@biomed.uib.no)

The nucleotide sequence data reported for the *cat-2* gene will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number HQ268827.

its genome, has several putative phosphorylation sites in the regulatory domain [20]. Thus phosphorylation appears to be a well-conserved mechanism regarding catecholamine synthesis regulation, at least at the level of amino acid sequence.

In the nematode *Caenorhabditis elegans*, a putative gene encoding TH was found and annotated as *cat-2* (abnormal catecholamine distribution). GFP (green fluorescent protein) expression analysis of the *cat-2* gene showed that it was expressed in eight neurons in the head and middle body of the hermaphrodite, and six extra neurons in the tail of the male [21]. These cells were reported to contain dopamine and were classified as sensory neurons [22]. Moreover, in the nematode, dopamine is the only catecholamine synthesized (no adrenaline and/or noradrenaline have been detected so far) [23]. Experiments with a *cat-2*-deletion mutant revealed that TH is required for the mechanosensation of the availability of food in the environment and for the correct locomotion in response to food [24]. More recent studies have shown that dopamine also exerts other functions, such as behavioural adaptation to different stimuli and learning [25,26].

In the present paper, we report the cloning of the *C. elegans cat-2* cDNA and the biochemical characterization of its encoded protein, CAT-2. CAT-2 shows TH activity and is phosphorylated by PKA at Ser^{35}, as identified by MS analysis. The enzyme, however, only shows a slight inhibitory effect by dopamine, and does not display the complex regulation characteristic of mammalian TH.

MATERIALS AND METHODS

C. elegans strains

The strain used in the present study was wild-type N2 Bristol. Maintenance and genetic manipulation of the nematode worms were performed following standard techniques [27]. Animals were grown at 20 °C, unless noted otherwise.

cDNA cloning of *C. elegans cat-2* gene

Information available at http://www.wormbase.org identifies the gene B0432.5/*cat-2* as encoding a putative TH, with two different spliced isoforms, B0432.5a and B0432.5b. The following primers were designed based on the genomic sequence: B0432.5a forward, 5′-GGAAATGTTTTTGCGGTAT-3′, and B0432.5a reverse, 5′-TCAATGTGCTCATCACTAGC-3′, for the amplification of isoform B0432.5a and B0432.5b forward, 5′-CAGATGAGGTGTCAGAAGGT-3′, and B0432.5b reverse, 5′-GGTTTCACATTGTAATCGATATT-3′, for the amplification of isoform B0432.5b. B0432.5a forward and B0432.5b reverse were also used to verify the possibility of one unique isoform, a combination of a and b which we referred to as isoform c (B0432.5c), although successful amplification of this new isoform was only achieved using a forward primer based on the sequence of SL1 (*trans*-splicing signal 1) 5′-GTTTAATTA-CCCAAGTTTGAG-3′, and B0432.5b reverse.

Total RNA from heterogeneous cultures of *C. elegans* was extracted following standard methods and using the TRIzol® reagent (Invitrogen). RT (reverse transcription)–PCR was performed using the primers mentioned above and the corresponding cDNA was cloned into a TOPO vector (Invitrogen) and sequenced.

Cloning and production of recombinant CAT-2 and hTH1

The *cat-2c* coding region (corresponding to isoform B0432.5c) was amplified from TOPO/cat-2c plasmid using the following primers, cat-2c_GSTforward, 5′-AATTCCCCGGGTATGTCG-TCACTAACCAAC-3′, and cat-2c_GSTreverse, 5′-TTGAG-CTCGAGTCATCACATTGTAATCGATA-3′, and cloned into the SmaI/XhoI sites of the pGEX4T-1 vector. The resulting pGEX4T-1/cat-2c construct was transformed into *Escherichia coli* strain BL21(DE3) pLysS cells (Invitrogen). Recombinant protein production was induced with 1 mM IPTG (isopropyl β-D-thiogalactopyranoside) for 6 h at 15 °C. The resulting fusion protein [GST (glutathione transferase)–CAT-2] was loaded on to a GST–Sepharose 4B column (GE Healthcare), and finally eluted with 10 mM glutathione. GST–CAT-2 was cleaved with thrombin (20 units/mg of fusion protein) at 4 °C for 16 h. The cleaved protein was purified further from thrombin and GST using concentration filters of 100 kDa cut-off (Amicon ultracentrifugal filters, Millipore), and the remaining fusion protein and GST were separated from CAT-2 with a second GST–Sepharose purification column. Purified CAT-2 was frozen immediately in liquid nitrogen until use. Protein concentration was measured using the Bio-Rad Laboratories protein assay kit, based on the Bradford assay [28].

Recombinant hTH1 was expressed in *E. coli* and purified as described previously [29].

Enzymatic activity assays

TH activity was measured at 25 °C using the method of Reinhard et al. [30] with minor modifications. The incubation mixture contained 100 mM sodium Hepes (pH 7.0), 50 μM L-[3,5–^{3}H]tyrosine (GE Healthcare), 0.5 mg/ml catalase and 20 μM $FeSO_4$ in a total volume of 100 μl. CAT-2 and hTH1 (1–3 μg) were pre-incubated for 1 min in this mixture, and the reaction was then triggered by addition of 500 μM BH_4 and 5 mM DTT (dithiothreitol). The reaction was stopped after 5 min by the addition of a slurry of activated charcoal in 1 M HCl. Samples were centrifuged at 11 000 ***g*** for 10 min, and aliquots of the supernatant were separated and counted in a scintillation counter. The steady-state kinetic parameters for the substrate L-tyrosine and the cofactor BH_4 were estimated with 50 μM L-tyrosine and various cofactor concentrations (0–1 mM) or at fixed concentrations of BH_4 (500 μM) and various concentrations of L-tyrosine (0–1 mM) respectively. The kinetic parameters were estimated by fitting to sigmoidal (for BH_4) or hyperbolic (for L-tyrosine) equations including substrate inhibition using SigmaPlot v.9.0. (Systat Software).

PAH and TPH activities were measured essentially as described for *C. elegans* PAH [31], using 100 mM sodium Hepes (pH 7.0), 0.5 mg/ml catalase, 20 μM $FeSO_4$, 0.1–5 mM either L-phenylalanine or 0.1–5 mM L-tryptophan, 0.5 mM BH_4 and 5 mM DTT, and a reaction time of 5 min at 25 °C.

Catecholamine inhibition was analysed by pre-incubating the enzyme in the incubation mixture with different concentrations of dopamine (0–100 μM) for 3 min, and then TH activity was measured as described above. The specific activity as a function of dopamine concentration was fitted to a hyperbolic decay with two parameters, the IC_{50} and the amplitude of inhibition.

Phosphorylation of CAT-2

Phosphorylation was performed at 30 °C with CAT-2 (10–20 μM subunit) in the presence of 500 μM of ATP, 10 mM $MgCl_2$ and 2.5 units of the catalytic subunit of PKA (New England BioLabs). Time-dependent phosphorylation was measured by taking aliquots at different times, and running these samples on SDS/PAGE (10% gels). Gels were stained with Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) following

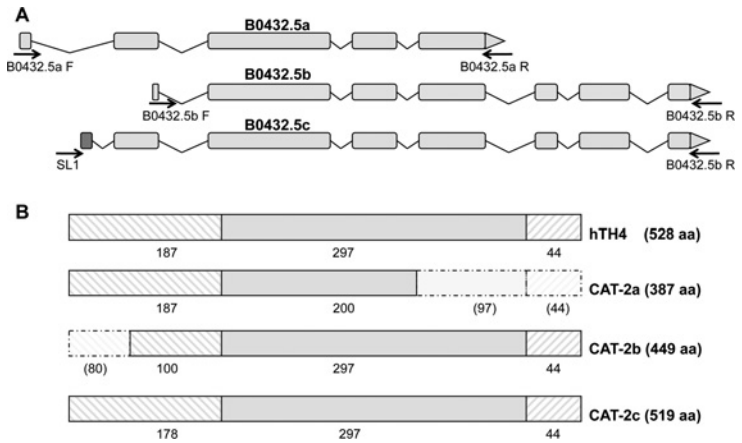

Figure 1 Representation of the *cat-2* gene and domain organization of the CAT-2 protein

(**A**) ORF of B0432.5 gene, with the three putative encoded isoforms. Grey boxes represent identified exons in Wormbase, the dark grey box represents the novel exon identified in the present study, and black lines represent introns. Arrows indicate the position and primers used to amplify the corresponding *cat-2* isoforms. (**B**) Domain organization of hTH4, and the different isoforms of CAT-2. Downward hatching indicates the regulatory domain, grey colour indicates the catalytic domain, and upward hatching indicates the oligomerization domain. In lighter colours and broken lines and parts of the protein that are not present in the corresponding isoforms.

the manufacturer's protocol. Subsequent regular staining with Coomassie Blue was performed to quantify total protein.

To measure the enzymatic parameters for the phosphorylated enzyme, CAT-2 was incubated for 5 min with PKA and ATP, and the phosphorylation reaction was stopped by loading the samples in a size-exclusion mini-column for buffer exchange; protein concentration was then recalculated. Corresponding non-phosphorylated control samples were treated under identical conditions as phosphorylated enzyme, except for the absence of PKA in the incubations, and the enzyme kinetic parameters were measured as described above.

Study of phosphorylated CAT-2 by MS analysis

Proteins in the excised bands were reduced, alkylated and in-gel trypsinized following standard procedure [32]. The proteolytic reaction was stopped by acidification with TFA (trifluoroacetic acid). Peptides from in-gel digestions were extracted and prepared for LC (liquid chromatography)–MS/MS (tandem MS) analysis. Microcolumns made by ourselves were used to filter the peptide samples in order to eliminate any gel particles. Samples were applied to the microcolumns pre-equilibrated in 60 % acetonitrile and 0.1 % TFA, filtered and collected.

After removal of acetonitrile by speed vacuum, the samples were injected into a nanoflow HPLC system online with positive electrospray ionization on a Ultima Global QTOF (quadrupole time-of-flight) instrument. MS analysis was performed essentially as described in [33] with minor modifications. The MS and MS/MS data generated from the nano-LC-QTOF were processed further using MassLynx 4.1 ProteinLynx software, and the in-house-developed software PklFileMerger. The Mascot generic files were processed using an in-house Mascot server (version 2.2) (Matrix Sciences) and searched against the *C. elegans* database, modified by addition of in-house CAT-2 sequence cloned in this work. Trypsin was selected as the enzyme allowing for one missed cleavage. Mascot was searched with a fragment mass tolerance of 0.2 Da and a parent ion tolerance of 0.3 Da. Scaffold (version Scaffold_2_06_01, Proteome Software) was used to validate MS/MS-based peptide and protein identifications. The post-translational modifications assigned were evaluated manually.

RESULTS

The B0432.5/*cat-2* gene encodes *C. elegans* TH

The current information available at Wormbase on the B0432.5/*cat-2* gene does not appear reliable as the data indicate that *cat-2* encodes two different isoforms: (i) B0532.5a, which has five exons encoding a putative TH protein that lacks an important part of the catalytic domain and the oligomerization domain, and (ii) B0532.5b, which encodes a protein with truncated regulatory domain, but with complete catalytic and oligomerization domains (Figure 1). A sequence alignment with other THs from vertebrates and invertebrates shows that these putative isoforms a and b do not match the archetypal domain organization of TH, that includes regulatory, catalytic and oligomerization domains (Figure 1B, and see Supplementary Figure S1 at http://www.BiochemJ.org/bj/434/bj4340133add.htm), strongly suggesting that the *cat-2* gene annotation at Wormbase might be incorrect. In turn, we hypothesized the existence of a different and unique isoform, a combination of a and b, that we referred to as B0532.5c (Figure 1). We then sequenced completely the only two ESTs (expressed sequence tags) available for the *cat-2* gene, yk806g04 and yk1013h05 (kindly provided by Dr Yuki Kohara, National Institute of Genetics, Mishima, Japan). The yk806 sequence corresponds to isofom b sequence, whereas, interestingly, the yk1013 sequence appears to be an immature mRNA of isoform c, supporting our hypothesis of the existence of the new isoform c, a combination of the other two (results not shown).

In order to demonstrate the existence of the c isoform, we isolated total RNA from a non-synchronized population of N2 wild-type *C. elegans* and synthesized the corresponding cDNA. We then used different sets of primer combinations to amplify each one of the three predicted isoforms using RT–PCR: B0432.5a forward and B0432.5a reverse for isoform a, B0432.5b forward and B0432.5b reverse for isoform b, and B0432.5a forward, SL1-based primer and B0432.5b reverse for the new isoform c (Figure 1A). We were not able to detect the a isoform after the PCR amplification, but we clearly detected the 1.6 kb band of c isoform and a band of 1.4 kb corresponding to b isoform, which is indeed included in the c isoform (see Supplementary Figure S2 at http://www.BiochemJ.org/bj/434/bj4340133add.htm). This result provides clear evidence of the existence of isoform B0532.5c in

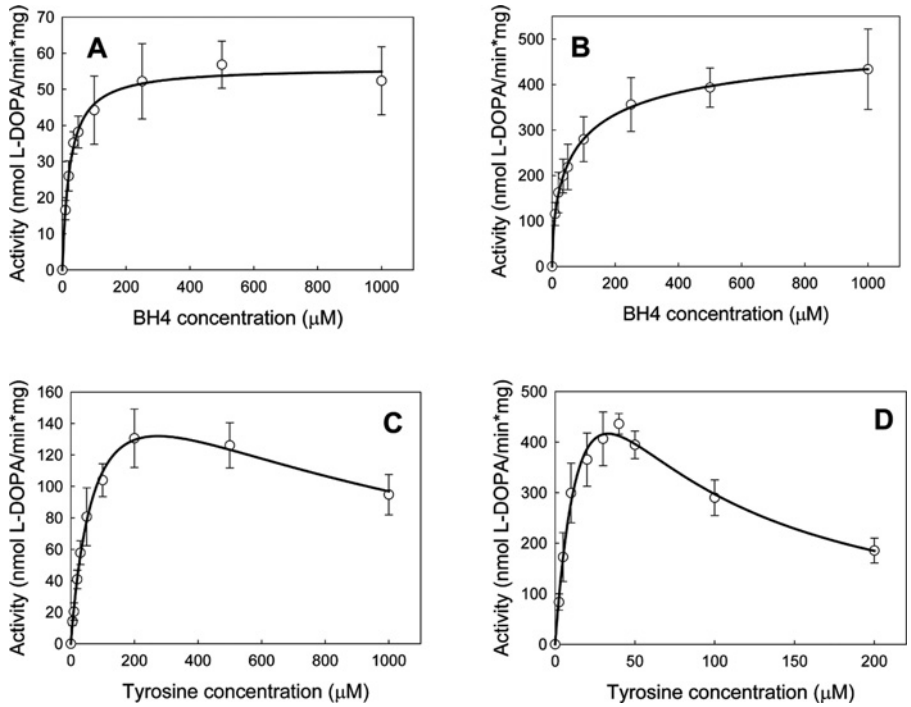

Figure 2 Enzymatic characterization of CAT-2 compared with hTH1

Steady-state kinetics of CAT-2 (**A** and **C**) and hTH1 (**B** and **D**). (**A** and **B**) Specific activity of CAT-2 and hTH1 at different concentrations of the cofactor (0–1000 μM) and fixed L-tyrosine concentration (50 μM). (**C** and **D**) Specific activity measured at different concentrations of the substrate (0–1000 μM for CAT-2 or 0–200 μM for hTH1) and fixed BH_4 concentration (500 μM). Results are means $\pm$ S.D. for three different experiments.

Table 1 Steady-state kinetic parameters for recombinant hTH1 and CAT-2

Results are means $\pm$ S.D. for three different experiments.

	BH_4*			L-Tyrosine†		
Enzyme	$S_{0.5}$ (μM)	Hill coefficient (h)	V_{max} (nmol of L-dopa/min per mg)	$S_{0.5}$ (μM)	K_i‡ (μM)	V_{max} (nmol of L-dopa/min per mg)
hTH1	83 $\pm$ 11	0.60 $\pm$ 0.03	531 $\pm$ 17	25 $\pm$ 6	45 $\pm$ 11	1038 $\pm$ 155
CAT-2	23 $\pm$ 2	1.03 $\pm$ 0.13	56 $\pm$ 2	83 $\pm$ 9	907 $\pm$ 128	212 $\pm$ 12

*Assayed with 0.05 mM L-tyrosine and 0–1 mM BH_4.
†Assayed with 0.5 mM BH_4 and 0–1 mM L-tyrosine for *C. elegans* TH, and 0–0.2 mM of L-tyrosine for hTH.
‡The concentration of L-tyrosine for half maximal substrate inhibition.

the nematode and we therefore focused on the cloning, expression and purification of this isoform.

Cloning of B0532.5c gene and recombinant protein production of CAT-2

To analyse the 5′-UTR (untranslated region) of the gene, we used RT–PCR with specific primers based on SL1 and SL2, which are predicted to be present in the 5′-UTR in 70% of the mRNAs in *C. elegans* [34]. With SL1 and B0432.5b reverse, we detected an mRNA with 65 nt in the 5′-UTR, 1557 nucleotides in the ORF (open reading frame) encoding eight exons (Figure 1A) and a 3′-UTR of 97 bp (based on information posted in Wormbase).

We cloned the coding region of *cat-2c* into the expression vector pGEX-4T-1 in order to be able to express and purify the recombinant protein (from now on just referred to as CAT-2) as a fusion protein with GST. Low temperatures (15 °C) and short induction durations (5–6 h) were used to significantly improve the yield of isolated protein (Supplementary Figure S2).

Catalytic and regulatory properties of CAT-2

We next studied the catalytic and regulatory properties of CAT-2, compared with the hTH1 (Table 1 and Figure 2). hTH1 is regulated by the natural cofactor BH_4, which binds with negative co-operativity [Hill coefficient (h) < 1] [35], whereas CAT-2 is devoid of this regulatory mechanism ($h \approx 1$). The $S_{0.5}$ constant for the cofactor is lower in CAT-2 (Table 1 and Figures 2A and 2B), reflecting a higher affinity for the cofactor. hTH1 activity-dependence on substrate (L-tyrosine) concentration shows substrate inhibition at high concentrations (>50 μM) of the substrate and a relatively low $S_{0.5}$, indicating high apparent affinity. CAT-2 also shows substrate inhibition, but at a higher concentration than for hTH1, compatible with a lower affinity for L-tyrosine (Table 1 and Figures 2C and 2D).

Another important mechanism regulating mammalian TH activity is the feedback inhibition by catecholamines, the end-products of the biosynthetic pathway [12,36] and we investigated the inhibitory effect of dopamine on CAT-2 activity. After incubation with different concentrations of dopamine at neutral

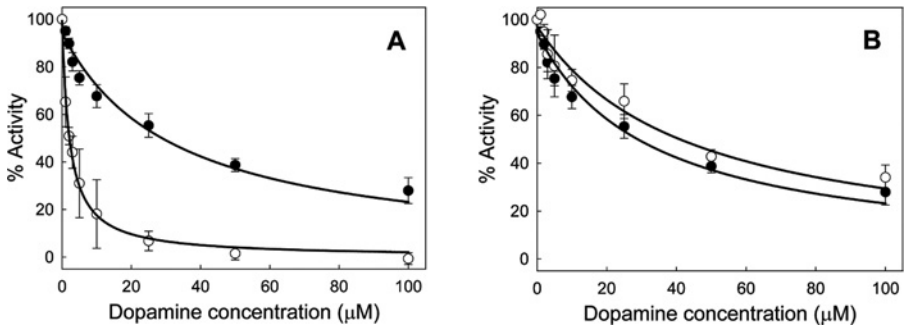

Figure 3 Inhibition by catecholamines

(**A**) TH residual activity of hTH1 (○) and CAT-2 (●) in the presence of different concentrations of dopamine. The lines represent the fitting of the experimental data to hyperbolic functions. (**B**) Dopamine inhibition in non-phosphorylated (●) and phosphorylated (○) CAT-2. Results are means ± S.D. for three different experiments.

Table 2 Substrate specificity of hTH1 and CAT-2

Activity was measured at 25 °C, with 0.5 mM BH_4.

Substrate (mM)	CAT-2 (nmol of product/min per mg)	hTH1 (nmol of product/min per mg)
L-Phenylanine (PAH activity)		
0.1	0.4 ± 0.1	337 ± 3
1	6.6 ± 2.8	714 ± 1
5	6.8 ± 0.1	851 ± 1
L-Tyrosine (TH activity)		
0.05	16.3 ± 12.1	555 ± 45
0.2	41.1 ± 10.9	272 ± 61
1	68.8 ± 0.6	275 ± 6
L-Tryptophan (TPH activity)		
0.1	<0.05	3.2 ± 0.1
1	0.3 ± 0.1	11.1 ± 0.1

pH and in the presence of iron, we measured the remaining activity in CAT-2 and hTH1. The human enzyme was inhibited with high affinity by the product ($IC_{50} \approx 2.2 \pm 0.1$ μM), whereas CAT-2 needed a higher concentration of dopamine to display a similar degree of inhibition ($IC_{50} \approx 32.6 \pm 4.6$ μM) (Figure 3A).

As a final step in the kinetic characterization, we investigated the substrate specificity; the human enzyme has been reported to display a high PAH activity, and lower but detectable TPH activity [1,37]. We measured the ability to hydroxylate tyrosine, phenylalanine and tryptophan, comparatively for hTH1 and CAT-2. Whereas hTH1 is able to hydroxylate phenylalanine and tyrosine at similar rates, CAT-2 shows clear specificity for tyrosine (Table 2). Neither hTH1 nor CAT-2 presented high activity when using tryptophan as substrate at the selected conditions.

CAT-2 is phosphorylated by PKA

The alignment between CAT-2 and other THs reveals a putative phosphorylation site in CAT-2, Ser^{35} (Figure 4B), which appears to be a good equivalent to the Ser^{40} phosphorylation site in hTH1, as seen by the sequence alignment of the N-terminal region (Figure 4B). PKA is the enzyme that phosphorylates Ser^{40} in the human enzyme [13], so we selected this kinase as the putative candidate to phosphorylate CAT-2. Time-dependent phosphorylation assays revealed a high phosphorylation rate. In fact, experiments with phosphoprotein staining (pro-Q Diamond) (Figure 4A) confirmed that CAT-2 is maximally phosphorylated within 5 min of incubation with PKA.

MS was used to identify the PKA-dependent phosphorylation site on CAT-2. Purified CAT-2 phosphorylated *in vitro* with PKA was subjected to SDS/PAGE. The proteins were then digested with trypsin and analysed by nano-LC–MS/MS. Tryptic digestion resulted in identification of CAT-2 protein with 99 % confidence (protein identification probability). Peptide 33–48 from CAT-2 with the sequence RYSLVHQASCETQHHK (Figure 5) was detected with parent ion masses *m*/*z* 1979.99 and *m*/*z* 2059.79, showing a difference of 80 Da between the non-phosphorylated and phosphorylated forms, which corresponds typically to the mass of a phosphate group less water, indicating the presence of a phosphopeptide. The MS/MS analysis identified further a peptide ion with *m*/*z* 515.95, corresponding to the quadruply charged singly phosphorylated CAT-2 peptide RYSLVHQASCETQHHK, which fragmented as illustrated by the spectrum shown in Figure 5(B). Data interpretation of the fragmentation spectra with detection of *y*-ions and *b*-ions together with the neutral loss fragment of the precursor ion strongly suggested that the peptide is phosphorylated, and that phosphate is positioned on Ser^3. Indeed, in experiments performed on a QTOF mass spectrometer in positive-ion mode, it was noted that this peptide underwent a gas-phase β-elimination reaction when subjected to CID (collision-induced dissociation), compatible with a neutral loss of phosphoric acid. Thus, in the MS/MS spectrum, a spacing of 69 Da corresponding to a diagnostic serine and owing to dehydroalanine formation was observed and indicated the exact location of the phosphorylated serine residue [38] (Figures 5A and 5B). The unique phosphosite at Ser^3 of the hexadecapeptide was assigned to Ser^{35} in *C. elegans* CAT-2 protein.

Activity measurements of phosphorylated CAT-2

CAT-2-P (Ser^{35}-phosphorylated CAT-2) showed similar specific activity and kinetic parameters for BH_4- and L-tyrosine-dependent TH activity as non-phosphorylated CAT-2 (results not shown). Non-phosphorylated CAT-2 and CAT-2-P were incubated with different amounts of dopamine, and the remaining activity was determined (Figure 3B). In contrast with the situation for hTH and other mammalian THs phosphorylated at Ser^{40} [39], phosphorylation in CAT-2 did not release the catecholamine inhibitory effect, since similar inhibitory rates were obtained. Thus the IC_{50} value is almost identical for both CAT-2-P ($\sim 43.4 \pm 6.7$ μM) and CAT-2 ($\sim 32.6 \pm 4.6$ μM).

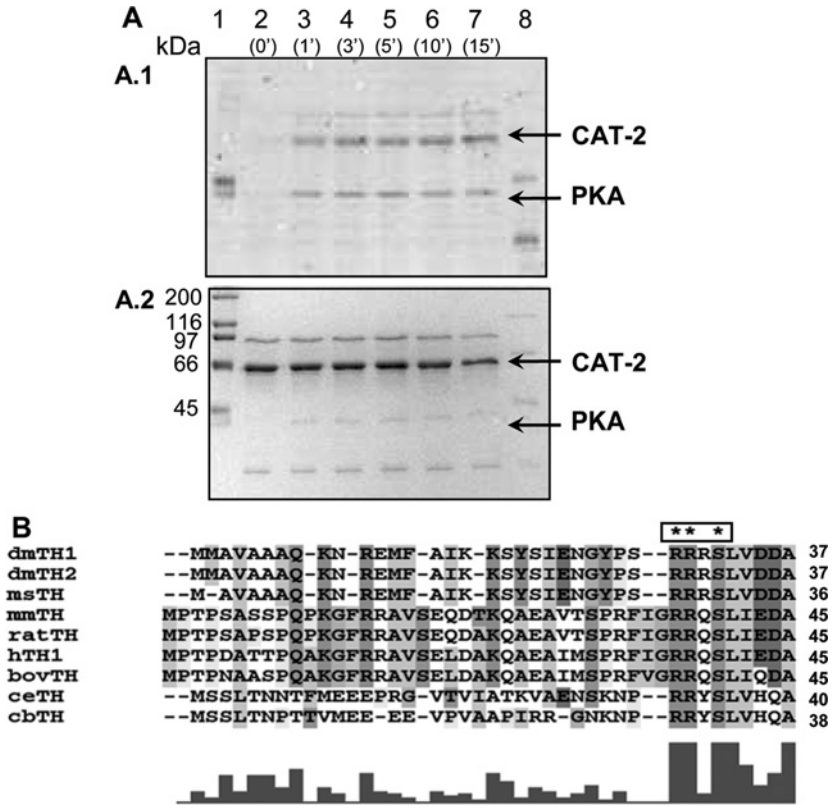

Figure 4 Phosphorylation of CAT-2 by PKA

(**A**) SDS/PAGE gels showing the time course phosphorylation of CAT-2 at 0 (lane 2), 1 (lane 3), 3 (lane 4), 5 (lane 5), 10 (lane 6) and 15 (lane 7) min. The molecular-mass standards for non-phosphorylated and phosphorylated proteins are shown in lanes 1 and 8 respectively, with sizes indicated in kDa. In A.1, the gel was stained with Pro-Q Diamond for phosphorylated proteins; in A.2, the same gel was stained with Coomassie Blue for total protein identification. Arrows point to CAT-2 (60 kDa) and PKA (38 kDa). (**B**) Alignment of the N-terminal domain sequence from several THs, from invertebrate and vertebrate organisms [mm (*Mus musculus*, NP_033403), rat (*Rattus norvegicus*, NP_036872), h (*Homo sapiens*, NP_000351), bov (*Bos taurus*, NP_776309), dm (*Drosophila melanogaster*, dmTH1, NP_476897 and dmTH2, NP_476898), ms (*Manduca sexta*, ABQ95973), ce (*Caenorhabditis elegans*) and cb (*Caenorhabditis briggsae*, XP_002632167)], showing putative phosphorylation sites. Note that the selection of the N-terminal region before alignment provides a different alignment within this area compared with the alignment when the whole sequences are analysed (see Supplementary Figure S1 at http://www.BiochemJ.org/bj/434/bj4340133add.htm). Alignments were performed using the program ClustalX 2.0.11.

DISCUSSION

In mammals, TH is the rate-limiting enzyme in the synthesis of catecholamines which takes place in the adrenal medulla and brain [40]. In the nematode *C. elegans*, dopamine is present in dopaminergic neurons, which were identified for the first time in the nematode by Sulston et al. [22] via the catecholamine-specific technique of FIF (formaldehyde-induced fluorescence). Mutant nematode worms for the *cat-2* gene were described as presenting dopamine-related defects, and they revealed almost complete loss of dopamine [22].

Every TH characterized so far has the typical three-domain organization described for the eukaryote AAAHs. The CAT-2 isoform c cloned and expressed in the present study also has this canonical three-domain organization (Figure 1B). In contrast, the annotated CAT-2 isoforms a and b (http://www.wormbase.org) appear to be immature or truncated variants of the wild-type CAT-2c protein. Consistently, we were unable to detect isoform a from total *C. elegans* mRNA, and the detected isoform b is likely to originate from the longer isoform c (Figure 1A).

The expressed and purified isoform c of CAT-2 displayed TH activity, with a specific activity of approx. 50 nmol of L-dopa/min per mg when measured under the standard assay conditions (50 μM L-tyrosine and 500 μM BH_4). This activity is lower than measured for the recombinant hTH1 (500−600 nmol of L-dopa/min per mg). On the other hand, the $S_{0.5}$ for the cofactor is lower for CAT-2 than for hTH1, reflecting a higher apparent affinity for BH_4 in the case of the nematode enzyme. More interestingly, CAT-2 is completely devoid of the negative co-operativity exerted by the cofactor on hTH, with an h of $\sim$1 for CAT-2 and $\sim$0.5 for hTH. The mechanisms for negative co-operativity are not as clear as for positive co-operativity, but, in the case of hTH, earlier results proposed that this regulatory mechanism might lead to a very high activity at low-micromolar levels of BH_4, which correspond to the BH_4 concentration in dopaminergic neurons [35]. CAT-2 does not appear to be regulated by the cofactor in this way, a property that could be related to the presence of higher concentrations of BH_4 in *C. elegans* neurons. Consistent with this, previous experiments from our laboratory showed that the total content of biopterin in nematode extracts is rather high, although no neuronal specific values were obtained [31]. On the other hand, the $S_{0.5}$ for the substrate is higher in CAT-2 than in hTH1 (approx. 3−4-fold), indicating lower apparent affinity for L-tyrosine. Accordingly, CAT-2 does not display the strong substrate inhibition manifested by hTH at L-tyrosine concentrations higher than 50 μM [41].

hTH has been reported to hydroxylate tyrosine and phenylalanine with the same efficiency, and also tryptophan to some extent [1,37]. In contrast, CAT-2 appears to be a more specific enzyme, since the rate of L-phenylalanine or L-tryptophan

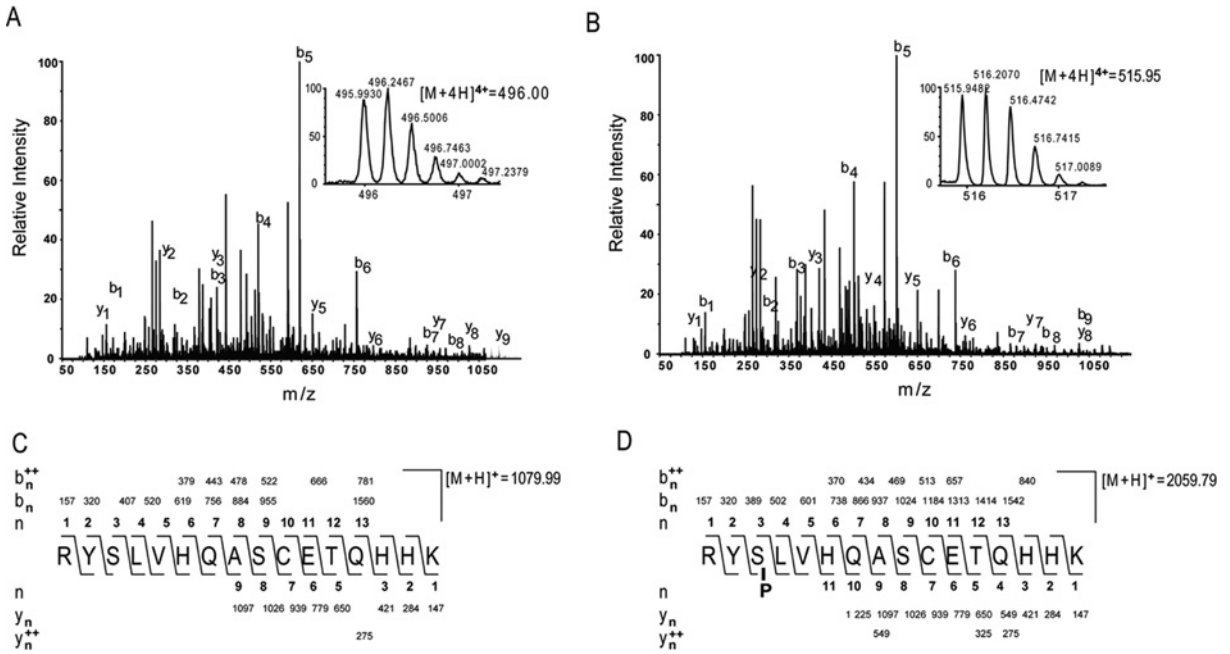

Figure 5 Fragmentation spectra of the non-phospho- and phospho-hexadecapeptide derived from the CAT-2 digestion

(**A** and **B**) Quadruply charged peptide at *m*/*z* 496.00 for the non-phosphoform (inset to **A**) and *m*/*z* 515.95 for the phosphoform (inset to **B**), detected by precursor scanning, were sequenced by MS/MS. Several fragment *y*-ion and *b*-ion series are observed (**A** and **B**), and, in addition, neutral loss of phosphate [− 98 (H_3PO_4)] is detected for b_3-ion (**B**). For the phosphopeptide, a *y*-ion series extending from y_1 to y_{10}, confirming the sequence QASCETQHHK is indicated. In addition, a complete *b*-ion series from b_1 to b_{13} was also observed within the spectrum corresponding to the sequence RYSpLVHQASCETQ. (**C** and **D**) Structures of the non-phospho- and phospho-hexadecapeptide as derived from the fragment signals. The peak masses matching the theoretical spectrum of the assigned peptide are indicated for various ion series (*b*, b^{++}, *y*, y^{++}). The spectra were produced from Mascot database searches with high confidence scores using data generated from a nano-LC-QTOF mass spectrometer.

hydroxylation is negligible when compared with L-tyrosine hydroxylation. Interestingly, the strict substrate specificity seems to be a characteristic of THs from lower eukaryotes, as a *D. melanogaster* neuronal isoform has also been reported to have high specificity for tyrosine hydroxylation [42].

The feedback catecholamine inhibitors bind to the active site of TH through co-ordination to the iron atom, which is oxidized to a ferric inactive state upon binding [43]. Phosphorylation by PKA reverts the enzyme to the active form by increasing the dissociation rate of catecholamines [12]. CAT-2 activity is decreased in the presence of dopamine, in a concentration-dependent manner, as happens with hTH, although the level of inhibition is significantly lower for the nematode protein. For the neuronal form of *D. melanogaster* TH, similar inhibition rates as CAT-2 have been reported, and, moreover, the epidermal isoform of the fruitfly protein is almost insensitive to dopamine inhibition [18]. These findings indicate that strong catecholamine inhibition might be a property of higher eukaryotes, which may be related with the increasing complexity of the nervous system in vertebrates, where neurotransmitter production has to be finely regulated.

hTH is phosphorylated at Ser^{40}, both *in vitro* and *in vivo*, by PKA [15], being by far the most studied and conserved phosphorylation site, as seen in the sequence alignment (Figure 4B). CAT-2 was phosphorylated at Ser^{35} by PKA, demonstrating that phosphorylation is a well-conserved regulatory mechanism in this enzyme, although the physiological consequences differ from that of the hTH. We hypothesized that, since CAT-2 is less inhibited by dopamine, the need to revert this inhibition is less critical in the nematode, as it is able to work efficiently even at high concentrations of dopamine. It would be interesting to study other putative roles of phosphorylation in CAT-2, such as interaction with partners. Bowling et al. [44] reported that, in *D. melanogaster*, TH interacts in a phosphorylation-dependent manner with GTP cyclohydrolase, the first enzyme in the synthesis pathway of the BH_4 cofactor. Moreover, in eukaryote organisms, the interaction between phosphorylated TH and 14-3-3 regulatory proteins is well established [45]. This interaction stimulates TH activity [46] and it has been shown recently that it can also regulate the distribution and localization of TH in cytosol and/or membranes from secretory granules [16]. *C. elegans* contains two genes coding for 14-3-3-like proteins, i.e. *par-5*/*ftt-1* and *ftt-2*, which are involved in cell polarity and lifespan through interaction with several protein partners [47]. It will be interesting to investigate the putative phosphorylation-dependent interaction of CAT-2 with *par-5* and *ftt-2*.

Altogether, our results indicate that CAT-2 is an enzyme that lacks, at least partially, some of the regulatory mechanisms present in the mammalian THs, such as negative co-operativity for BH_4, substrate inhibition and feedback inhibition by dopamine. We propose that CAT-2 does not need such a tight regulation as hTH and behaves more like a constitutively active enzyme, with lower but constant activity, as also suggested by Vie et al. [18] for the epidermal isoform of *D. melanogaster* TH.

The physiological relevance of a constitutively active TH is difficult to interpret without *in vivo* experimentation. There are nevertheless some functions of dopamine in the nematode that might support the need for an unregulated TH. Thus the requirement for dopamine to sense food sources is necessarily continuous, as it has been reported that *C. elegans* feeds until all food is depleted completely [24]. Constant production of dopamine could be advantageous for the survival and feeding of the nematode, both to ensure that *C. elegans* stays in the bacterial lawn and to help finding new food sources [24,48]. Moreover, the

characteristic complex regulation of mammalian TH seems to be related to a higher neuronal complexity in mammals. In humans, mutations in the *TH* gene lead to severe neurological diseases [49] and transgenic mice bearing a deletion in the *th* gene are severely impaired [50], and most are born already dead and/or with severe abnormalities. In contrast, *C. elegans cat-2* mutants are viable and apparently healthy [24], which may reflect a less critical role of TH in nematode survival and development, with reduced requirement for tight regulation of its enzymatic activity.

AUTHOR CONTRIBUTION

Ana Calvo, Antonio Miranda-Vizuete and Aurora Martinez designed the research; Ana Calvo, Angel Pey and Anne Døskeland performed experiments; Ana Calvo analysed data and all the authors contributed to discussion of results and to writing the paper.

ACKNOWLEDGEMENTS

We thank the *Caenorhabditis* Genetics Center (http://www.cbs.umn.edu/CGC/strains/acknowledge.html) for the nematode strains used in the present study, and to Dr. Yuki Kohara for kindly providing the ESTs. We are very thankful to Randi Svebak for expert technical help. The MS analyses were performed at the proteomic platform PROBE (FUGE, The Research Council of Norway) and we thank Frode Berven for helpful discussions.

FUNDING

This study was supported by The Research Council of Norway, the Meltzer Fond and the Instituto de Salud Carlos III [project numbers PI050065 and PI080557], co-financed with the Fondo Europeo de Desarrollo Regional (FEDER), and Junta de Andalucía [project numbers P07-CVI-02697 and P08-CVI-03629]. A.L.P. is a recipient of a Ramon y Cajal contract from the Spanish Ministry of Science and Innovation (MICINN).

REFERENCES

1 Teigen, K., McKinney, J. A., Haavik, J. and Martinez, A. (2007) Selectivity and affinity determinants for ligand binding to the aromatic amino acid hydroxylases. Curr. Med. Chem. **14**, 455–467
2 Fitzpatrick, P. F. (2000) The aromatic amino acid hydroxylases. Adv. Enzymol. Relat. Areas Mol. Biol. **74**, 235–294
3 Flatmark, T. and Stevens, R. C. (1999) Structural insight into the aromatic amino acid hydroxylases and their disease-related mutant forms. Chem. Rev. **99**, 2137–2160
4 Leiros, H. K., Pey, A. L., Innselset, M., Moe, E., Leiros, I., Steen, I. H. and Martinez, A. (2007) Structure of phenylalanine hydroxylase from *Colwellia psychrerythraea* 34H, a monomeric cold active enzyme with local flexibility around the active site and high overall stability. J. Biol. Chem. **282**, 21973–21986
5 Erlandsen, H., Kim, J. Y., Patch, M. G., Han, A., Volner, A., Abu-Omar, M. M. and Stevens, R. C. (2002) Structural comparison of bacterial and human iron-dependent phenylalanine hydroxylases: similar fold, different stability and reaction rates. J. Mol. Biol. **320**, 645–661
6 Chen, Y. C., Priyadarshini, M. and Panula, P. (2009) Complementary developmental expression of the two tyrosine hydroxylase transcripts in zebrafish. Histochem. Cell Biol. **132**, 375–381
7 Nagatsu, T. (1995) Tyrosine hydroxylase: human isoforms, structure and regulation in physiology and pathology. Essays Biochem. **30**, 15–35
8 Dumas, S., Le Hir, H., Bodeau-Pean, S., Hirsch, E., Thermes, C. and Mallet, J. (1996) New species of human tyrosine hydroxylase mRNA are produced in variable amounts in adrenal medulla and are overexpressed in progressive supranuclear palsy. J. Neurochem. **67**, 19–25
9 Ohye, T., Ichinose, H., Yoshizawa, T., Kanazawa, I. and Nagatsu, T. (2001) A new splicing variant for human tyrosine hydroxylase in the adrenal medulla. Neurosci. Lett. **312**, 157–60
10 Nishii, K., Matsushita, N., Sawada, H., Sano, H., Noda, Y., Mamiya, T., Nabeshima, T., Nagatsu, I., Hata, T., Kiuchi, K. et al. (1998) Motor and learning dysfunction during postnatal development in mice defective in dopamine neuronal transmission. J. Neurosci. Res. **54**, 450–464
11 Kobayashi, K. (2001) Role of catecholamine signaling in brain and nervous system functions: new insights from mouse molecular genetic study. J. Invest. Dermatol. Symp. Proc. **6**, 115–121
12 Kumer, S. C. and Vrana, K. E. (1996) Intricate regulation of tyrosine hydroxylase activity and gene expression. J. Neurochem. **67**, 443–462
13 Sura, G. R., Daubner, S. C. and Fitzpatrick, P. F. (2004) Effects of phosphorylation by protein kinase A on binding of catecholamines to the human tyrosine hydroxylase isoforms. J. Neurochem. **90**, 970–978
14 Ramsey, A. J. and Fitzpatrick, P. F. (1998) Effects of phosphorylation of serine 40 of tyrosine hydroxylase on binding of catecholamines: evidence for a novel regulatory mechanism. Biochemistry **37**, 8980–8986
15 Dunkley, P. R., Bobrovskaya, L., Graham, M. E., von Nagy-Felsobuki, E. I. and Dickson, P. W. (2004) Tyrosine hydroxylase phosphorylation: regulation and consequences. J. Neurochem. **91**, 1025–43
16 Halskau, Jr, O., Ying, M., Baumann, A., Kleppe, R., Rodriguez-Larrea, D., Almas, B., Haavik, J. and Martinez, A. (2009) Three-way interaction between 14-3-3 proteins, the N-terminal region of tyrosine hydroxylase, and negatively charged membranes. J. Biol. Chem. **284**, 32758–32769
17 Haycock, J. W. and Wakade, A. R. (1992) Activation and multiple-site phosphorylation of tyrosine hydroxylase in perfused rat adrenal glands. J. Neurochem. **58**, 57–64
18 Vie, A., Cigna, M., Toci, R. and Birman, S. (1999) Differential regulation of *Drosophila* tyrosine hydroxylase isoforms by dopamine binding and cAMP-dependent phosphorylation. J. Biol. Chem. **274**, 16788–16795
19 Gorman, M. J., An, C. and Kanost, M. R. (2007) Characterization of tyrosine hydroxylase from *Manduca sexta*. Insect Biochem. Mol. Biol. **37**, 1327–1337
20 Gaskell, E. A., Smith, J. E., Pinney, J. W., Westhead, D. R. and McConkey, G. A. (2009) A unique dual activity amino acid hydroxylase in *Toxoplasma gondii*. PLoS ONE **4**, e4801
21 Lints, R. and Emmons, S. W. (1999) Patterning of dopaminergic neurotransmitter identity among *Caenorhabditis elegans* ray sensory neurons by a TGFβ family signaling pathway and a *Hox* gene. Development **126**, 5819–5831
22 Sulston, J., Dew, M. and Brenner, S. (1975) Dopaminergic neurons in the nematode *Caenorhabditis elegans*. J. Comp. Neurol. **163**, 215–226
23 Nass, R. and Blakely, R. D. (2003) The *Caenorhabditis elegans* dopaminergic system: opportunities for insights into dopamine transport and neurodegeneration. Annu. Rev. Pharmacol. Toxicol. **43**, 521–544
24 Sawin, E. R., Ranganathan, R. and Horvitz, H. R. (2000) *C. elegans* locomotory rate is modulated by the environment through a dopaminergic pathway and by experience through a serotonergic pathway. Neuron **26**, 619–631
25 Sanyal, S., Wintle, R. F., Kindt, K. S., Nuttley, W. M., Arvan, R., Fitzmaurice, P., Bigras, E., Merz, D. C., Hebert, T. E., van der Kooy, D. et al. (2004) Dopamine modulates the plasticity of mechanosensory responses in *Caenorhabditis elegans*. EMBO J. **23**, 473–482
26 Kindt, K. S., Quast, K. B., Giles, A. C., De, S., Hendrey, D., Nicastro, I., Rankin, C. H. and Schafer, W. R. (2007) Dopamine mediates context-dependent modulation of sensory plasticity in *C. elegans*. Neuron **55**, 662–676
27 Brenner, S. (1974) The genetics of *Caenorhabditis elegans*. Genetics **77**, 71–94
28 Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**, 248–254
29 Haavik, J., Le Bourdelles, B., Martínez, A., Flatmark, T. and Mallet, J. (1991) Recombinant human tyrosine hydroxylase isozymes: reconstitution with iron and inhibitory effect of other metal ions. Eur. J. Biochem. **199**, 371–378
30 Reinhard, Jr, J. F., Smith, G. K. and Nichol, C. A. (1986) A rapid and sensitive assay for tyrosine-3-monooxygenase based upon the release of ^3H_2O and adsorption of [^{3}H]-tyrosine by charcoal. Life Sci. **39**, 2185–2189
31 Calvo, A. C., Pey, A. L., Ying, M., Loer, C. M. and Martinez, A. (2008) Anabolic function of phenylalanine hydroxylase in *Caenorhabditis elegans*. FASEB J. **22**, 3046–3058
32 Shevchenko, A., Tomas, H., Havlis, J., Olsen, J. V. and Mann, M. (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat. Protoc. **1**, 2856–2860
33 Birkeland, E., Nygaard, G., Oveland, E., Mjaavatten, O., Ljones, M., Doskeland, S. O., Krakstad, C. and Selheim, F. (2009) Epac-induced alterations in the proteome of human SH-SY5Y nueroblastoma cells. J. Proteomics Bioinform. **2**, 244–254
34 Graber, J. H., Salisbury, J., Hutchins, L. N. and Blumenthal, T. (2007) *C. elegans* sequences that control *trans*-splicing and operon pre-mRNA processing. RNA **13**, 1409–1426
35 Flatmark, T., Almås, B., Knappskog, P. M., Berge, S. V., Svebak, R. M., Chehin, R., Muga, A. and Martínez, A. (1999) Tyrosine hydroxylase binds tetrahydrobiopterin cofactor with negative cooperativity, as shown by kinetic analyses and surface plasmon resonance detection. Eur. J. Biochem. **262**, 840–849
36 Nakashima, A., Mori, K., Suzuki, T., Kurita, H., Otani, M., Nagatsu, T. and Ota, A. (1999) Dopamine inhibition of human tyrosine hydroxylase type 1 is controlled by the specific portion in the N-terminus of the enzyme. J. Neurochem. **72**, 2145–2153

37 Fukami, M. H., Haavik, J. and Flatmark, T. (1990) Phenylalanine as substrate for tyrosine hydroxylase in bovine adrenal chromaffin cells. Biochem. J. **268**, 525–528
38 Mann, M., Ong, S. E., Gronborg, M., Steen, H., Jensen, O. N. and Pandey, A. (2002) Analysis of protein phosphorylation using mass spectrometry: deciphering the phosphoproteome. Trends Biotechnol. **20**, 261–268
39 Haavik, J., Martínez, A. and Flatmark, T. (1990) pH-dependent release of catecholamines from tyrosine hydroxylase and the effect of phosphorylation of Ser-40. FEBS Lett. **262**, 363–365
40 Kaufman, S. (1995) Tyrosine hydroxylase. Adv. Enzymol. Relat. Areas Mol. Biol. **70**, 103–220
41 Quinsey, N. S., Luong, A. Q. and Dickson, P. W. (1998) Mutational analysis of substrate inhibition in tyrosine hydroxylase. J. Neurochem. **71**, 2132–2138
42 Neckameyer, W. S., Holt, B. and Paradowski, T. J. (2005) Biochemical conservation of recombinant *Drosophila* tyrosine hydroxylase with its mammalian cognates. Biochem. Genet. **43**, 425–443
43 Almås, B., Le Bourdelles, B., Flatmark, T., Mallet, J. and Haavik, J. (1992) Regulation of recombinant human tyrosine hydroxylase isozymes by catecholamine binding and phosphorylation: structure/activity studies and mechanistic implications. Eur. J. Biochem. **209**, 249–255
44 Bowling, K. M., Huang, Z., Xu, D., Ferdousy, F., Funderburk, C. D., Karnik, N., Neckameyer, W. and O'Donnell, J. M. (2008) Direct binding of GTP cyclohydrolase and tyrosine hydroxylase: regulatory interactions between key enzymes in dopamine biosynthesis. J. Biol. Chem. **283**, 31449–31459
45 Aitken, A. (2006) 14-3-3 proteins: a historic overview. Semin. Cancer Biol. **16**, 162–172
46 Kleppe, R., Toska, K. and Haavik, J. (2001) Interaction of phosphorylated tyrosine hydroxylase with 14-3-3 proteins: evidence for a phosphoserine 40-dependent association. J. Neurochem. **77**, 1097–1107
47 Wang, Y., Oh, S. W., Deplancke, B., Luo, J., Walhout, A. J. and Tissenbaum, H. A. (2006) *C. elegans* 14-3-3 proteins regulate life span and interact with SIR-2. 1 and DAF-16/FOXO. Mech. Ageing Dev. **127**, 741–747
48 Hills, T., Brockie, P. J. and Maricq, A. V. (2004) Dopamine and glutamate control area-restricted search behavior in *Caenorhabditis elegans*. J. Neurosci. **24**, 1217–1225
49 Haavik, J., Blau, N. and Thony, B. (2008) Mutations in human monoamine-related neurotransmitter pathway genes. Hum. Mutat. **29**, 891–902
50 Zhou, Q. Y., Quaife, C. J. and Palmiter, R. D. (1995) Targeted disruption of the tyrosine hydroxylase gene reveals that catecholamines are required for mouse fetal development. Nature **374**, 640–643

Received 24 September 2010/15 November 2010; accepted 18 November 2010
Published as BJ Immediate Publication 18 November 2010, doi:10.1042/BJ20101561

Biochem. J. (2011) **434**, 133–141 (Printed in Great Britain) doi:10.1042/BJ20101561

online data

SUPPLEMENTARY ONLINE DATA

Divergence in enzyme regulation between *Caenorhabditis elegans* and human tyrosine hydroxylase, the key enzyme in the synthesis of dopamine

Ana C. CALVO*[1], Angel L. PEY†, Antonio MIRANDA-VIZUETE‡§, Anne P. DØSKELAND* and Aurora MARTINEZ*[1]

*Department of Biomedicine, University of Bergen, Jonas Lies vei 91, N-5009 Bergen, Norway, †Departamento de Química Física, Facultad de Ciencias, Universidad de Granada, 18071-Granada, Spain, ‡Centro Andaluz de Biología del Desarrollo (CABD-CSIC), Departamento de Fisiología, Anatomía y Biología Celular, Universidad Pablo de Olavide, E-41013 Sevilla, Spain, and §Instituto de Biomedicina de Sevilla, Hospital Universitario Virgen del Rocío/CSIC/Universidad de Sevilla, E-41013 Sevilla, Spain

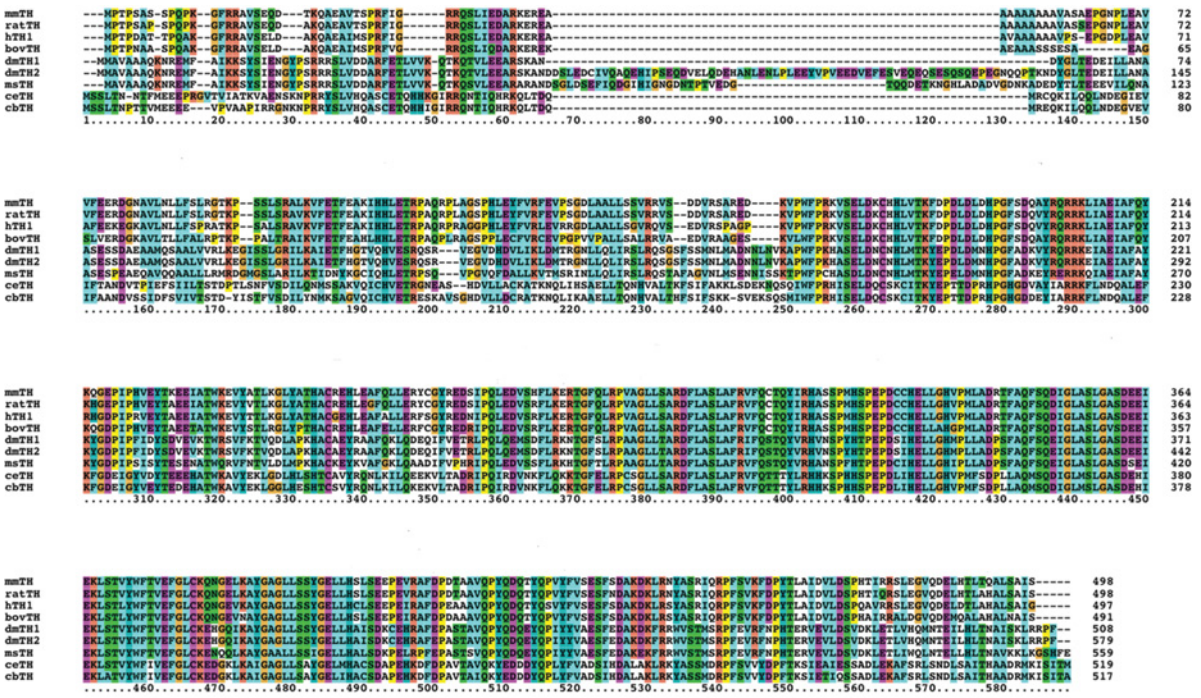

Figure S1 Alignment of several THs from invertebrate and vertebrate organisms

mm (*Mus musculus*, NP_033403), rat (*Rattus norvegicus*, NP_036872), h (*Homo sapiens*, NP_000351), bov (*Bos taurus*, NP_776309), dm (*Drosophila melanogaster*, dmTH1, NP_476897 and dmTH2, NP_476898), ms (*Manduca sexta*, ABQ95973), ce (*Caenorhabditis elegans*) and cb (*Caenorhabditis briggsae*, XP_002632167). Alignments were performed using the program ClustalX 2.0.11.

[1] Correspondence may be addressed to either of these authors (email ana.calvo@biomed.uib.no or aurora.martinez@biomed.uib.no)

The nucleotide sequence data reported for the *cat-2* gene will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under accession number HQ268827.

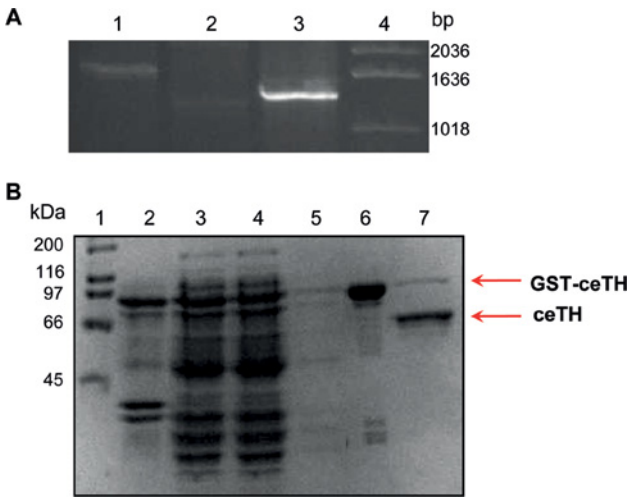

Figure S2 Cloning of the *cat-2* gene and purification of its corresponding protein CAT-2

(**A**) Agarose gel showing the RT–PCR amplification of the different putative isoforms encoded by *cat-2* gene B0432.5c (lane 1), B0432.5a (lane 2), B0432.5b (lane 3) and ladder (lane 4), with sizes indicated in bp. (**B**) SDS/PAGE gel showing expression in *E. coli* and purification of CAT-2. Some fusion protein is present in the pellet of the bacterial extract (lane 2), although the main fraction is in the soluble extract (lane 3). Different steps in the purification procedure on GST–Sepharose, flowthrough from the extract application (lane 4) and column wash (lane 5). Elution and concentration (lane 6) provide highly pure GST–CAT-2 fusion protein, and, after cleavage and repurification (lane 7), most of the fusion protein is removed. Molecular-mass standards are also shown (lane 1), with sizes indicated in kDa. ce, *C. elegans*.

Received 24 September 2010/15 November 2010; accepted 18 November 2010
Published as BJ Immediate Publication 18 November 2010, doi:10.1042/BJ20101561