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Purification and characterization of the blue–green rat phaeochromocytoma (PC12) tyrosine hydroxylase with a dopamine–Fe(III) complex

Reversal of the endogenous feedback inhibition by phosphorylation of serine-40

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Tyrosine hydroxylase (TH) was purified from tumours of rat phaeochromocytoma (PC12) cells by a three-step purification procedure giving 30 mg of pure enzyme in 3 days. The enzyme sedimented with an $s_{20,w}$ value of 9.2 S and revealed an apparent subunit molecular mass of 62 kDa with a minor 60 kDa component. Two-dimensional gel isoelectric focusing/electrophoresis and tryptic digestion revealed that the heterogeneity could be accounted for by limited proteolysis of the 62 kDa component and the presence of covalently bound phosphate. The enzyme had a strong blue–green colour ($\epsilon_{700} = 3.1 \pm 0.2$ mM-iron$^{-1} \cdot$cm^{-1}). The resonance Raman spectrum obtained with $\lambda_{excitation} = 605$ nm revealed the presence of an Fe(III)–catecholamine complex in the isolated enzyme, similar to that observed in the bovine adrenal enzyme [Andersson, Cox, Que, Flatmark & Haavik (1988) J. Biol. Chem. **263**, 18621–18626]. In the rat PC12 enzyme, all of the iron present (0.53 ± 0.03 atom per subunit) seems to be chelated by the feedback inhibitors (0.49 ± 0.05 mol of dopamine and 0.10 ± 0.03 mol of noradrenaline per mol of subunit). The e.p.r. spectra at 3.6 K show g-values at 7.0, 5.2 and 1.9 as observed for other catecholate-complexed enzymes. After phosphorylation of serine-40 and addition of L-tyrosine a new rhombic ($|E/D| = 0.33$) e.p.r. species could be observed. Phosphorylation of serine-40 by cyclic AMP-dependent protein kinase increased the catalytic activity; depending on assay conditions, up to 80–110-fold activation could be observed when measured at high TH (i.e. high endogenous catecholamine) concentration.

INTRODUCTION

Tyrosine hydroxylase (TH), phenylalanine hydroxylase (PAH) and tryptophan hydroxylase (TRPH) constitute a group of mammalian mono-oxygenases which all require iron and a tetrahydrobiopterin cofactor for their catalytic activity (Kaufman & Fisher, 1974). They utilize O_2 to oxidize the reduced pterin to a labile 4α-hydroxytetrahydrobiopterin (4a-carbinolamine) which dehydrates to dihydropterin (Lazarus *et al.*, 1982; Dix *et al.*, 1987; Haavik & Flatmark, 1987; Dix & Benkovic, 1988). The recent cloning and sequencing of cDNAs for these hydroxylases have revealed major areas of sequence identity in the presumed catalytic (*C*-terminal) domain (Grenett *et al.*, 1987).

Tyrosine 3-mono-oxygenase (tyrosine hydroxylase, EC 1.14.16.2) catalyses the hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (dopa). This reaction is the rate-limiting step (Nagatsu *et al.*, 1964) in the biosynthesis of catecholamines, which serve important biological functions as neurotransmitters or hormones. The catecholamine biosynthesis is probably short-term, on the time scale of minutes, regulated by feedback inhibition (Udenfriend *et al.*, 1965) and phosphorylation/dephosphorylation of TH by specific protein kinases and phosphatases (Ames *et al.*, 1977; Kaufman & Kaufman, 1985; Andersson *et al.*, 1989*a*; Haavik *et al.*, 1989*b*; Zigmond *et al.*, 1989).

The difficulty in preparing TH has long been an obstacle to the physico-chemical studies of this enzyme, which is considered an important marker of cellular differentiation in the nervous system (i.e. catecholaminergic neurons). Recently the rat enzyme has been cloned and expressed in insect tissue-culture cells (Fitzpatrick *et al.*, 1990); likewise the isoforms of human TH have been cloned and expressed in eukaryotic cells (Horellou *et al.*, 1989) and *Escherichia coli* (Haavik *et al.*, 1991). Studies of the rat enzyme expressed in the baculovirus system (Fitzpatrick, 1989; Fitzpatrick *et al.*, 1990) and purified PC12 TH from cell cultures (Dix *et al.*, 1987) indicates that the rat enzyme has physico-chemical properties which are different from the purified bovine adrenal TH (Haavik *et al.*, 1988). Both these preparations of rat enzyme lack the characteristic blue–green colour found in the bovine TH, which is due to an adrenaline(noradrenaline)–Fe(III) complex (Andersson *et al.*, 1987, 1988). The reported differences between the rat and bovine TH could indicate differences in their iron-binding and/or active sites, although rat and bovine TH have a predicted amino acid sequence similarity in the regulatory domain of 66% (amino acids 1–157) and in the catalytic domain of 91% (amino acids 158–491) (Saadat *et al.*, 1988). Phosphorylation of rat PC12 TH by different protein kinases has been shown to occur at Ser-8, Ser-19, Ser-31, Ser-40 (and Ser-153); Ser-40 can be phosphorylated by several kinases (Campbell *et al.*, 1986; Pigeon *et al.*, 1987; Haycock, 1990). The affinity of catecholamines to enzyme-bound Fe(III) can be modulated by specific phosphorylation of Ser-40 in bovine TH (Andersson *et al.*, 1989*a*; Haavik *et al.*, 1990) and low pH dissociates the feedback inhibitors from the enzyme (Haavik *et al.*, 1990).

Abbreviations used: TH, tyrosine hydroxylase; PAH, phenylalanine hydroxylase; TRPH, tryptophan hydroxylase; dopa, dihydroxyphenylalanine; 6-MePH$_4$, 6-methyltetrahydropterin; PB, phosphate buffer; PDA, *N*-carboxymethyl-*N*-2-picolylglycine.

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The adrenal medulla is the tissue with the highest TH content, and for this reason the adrenal gland or tumours derived from it have frequently been used for the isolation of TH. Here we describe a new improved scaled-up rapid purification procedure of TH from rat pheochromocytoma tumours, originally developed, but not described, by Thibault *et al.* (1981). The present study was performed in order to compare the native phosphorylated and non-phosphorylated TH from transplanted phaeochromocytoma (PC12) tumours, the rat TH expressed in insect tissue-culture cells as well as the bovine adrenal enzyme. In a preliminary account of this work we showed that PC12 TH as isolated contained dopamine and had blue–green colour similar to that of bovine TH (Andersson *et al.*, 1989*b*). We now show that the tumour derived form of rat TH is highly active, is not processed and has a similar iron content and iron-binding site as the bovine enzyme, in contrast with previous reports of the rat enzyme.

EXPERIMENTAL

Animals and tumours

The PC12 cell clone isolated by Greene & Tischler (1976) from a rat phaeochromocytoma tumour (Warren & Schute, 1972), was a gift from Dr. H. Thoenen (Max Planck Institute for Psychiatry, Martinsried, Germany). Cells were cultivated *in vitro* as described (Greene & Tischler, 1977) and collected in sterile iso-osmotic buffer [20 mM-potassium/sodium phosphate (pH 7.2)/0.150 M-NaCl]. Cultured PC12 cells (5×10^6) were injected subcutaneously in 2-month-old rats of the New England Deaconess Hospital strain. After 3 weeks, the resulting tumours (average weight 10 g) were removed surgically from CO_2-asphyxiated rats. The surrounding fibrous tissues were removed and one portion of the tumours was frozen in liquid N_2 and stored at −80 °C. The other part was dispersed by mechanical stirring with a Pasteur pipette in sterile iso-osmotic buffer (1 g of tumour per 10 ml of medium) and new animals were injected with 1 ml of this cell suspension.

Enzymes, substrates and chemicals

Standard chemicals were all of the highest purity commercially available. Molecular-mass markers were from Boehringer (Mannheim, Germany). Catalase, alcohol dehydrogenase and chymotrypsin were from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Octopine dehydrogenase was a gift from Dr. A. Olomukj, Collège de France, Paris, France; 6,7-dimethyltetrahydropterin and 6-methyltetrahydropterin (6-MePH_4) were from Calbiochem (La Jolla, CA, U.S.A.). [3,5-^{3}H]Tyrosine from Amersham International was purified just before use by percolation on alumina, followed by chromatography on a Dowex 50 (Bio-Rad) column (Ikeda *et al.*, 1966). The hydrophobic chromatography support was phenyl-Sepharose (Pharmacia, Sweden). IBF (Villeneuve-la-Garenne, France) laboratory provided us with heparin–Ultrogel and hydroxyapatite–Ultrogel.

Assay of TH activity

TH activity was routinely assayed as described by Levitt *et al.* (1976), using 60 μM-L tyrosine and 720 μM-6,7-dimethyl-5,6,7,8-tetrahydropterin. One unit of TH corresponds to 1 nmol of L-dopa formed/h at 37 °C at pH 6.5 [no corrections were made for the so-called NIH-shift (see Dix & Benkovic, 1988)]. For the purpose of comparison with the bovine enzyme, TH was also assayed by an alternative method based on h.p.l.c. (Haavik & Flatmark, 1980; Haavik *et al.*, 1988) at 30 °C in 0.1 M-potassium phosphate, pH 7.2, in the presence of catalase with 0.5 mM-6-MePH_4 and 20 μM-L-Tyr or 20 μM-L-Phe; no extra iron was added. Activation by phosphorylation under e.p.r. conditions was determined as described by Andersson *et al.* (1989*a*). Protein concentrations were determined by the Bradford (1976) method with BSA as a standard.

TH purification

The three week old tumours were frozen in liquid N_2, then ground at −20 °C into a fine powder with a Waring Blendor at maximum speed for 1 min. The frozen tumour powder (100 g) was suspended in three times its weight of cold 20 mM-potassium phosphate buffer, pH 7.2 (PB), supplemented with 1 mM-phenylmethanesulphonyl fluoride and homogenized for 1 min in a Waring Blendor at maximum speed. All remaining steps were performed at 4 °C. The homogenate was centrifuged first at 15000 *g* for 30 min, then at 150000 *g* for 2 h. The 150000 *g*/2 h supernatant (S 150) was precipitated by the addition of solid $(NH_4)_2SO_4$ to give a final saturation of 50%. At 1 h after final solubilization of the salt, the precipitated protein was recovered by centrifugation at 15000 *g* for 30 min. The pellet was dissolved by addition of three volumes of the PB containing $(NH_4)_2SO_4$ at 20% saturation. After 1 h of gentle stirring, the insoluble material was removed by centrifugation at 10000 *g* for 30 min and the supernatant was applied directly to a phenyl-Sepharose column.

Hydrophobic affinity chromatography. The sample containing the resolubilized proteins was loaded on to a phenyl-Sepharose column (4 cm × 80 cm), equilibrated with 20% saturation of $(NH_4)_2SO_4$ in PB. Subsequently, the column was washed with 1 litre of 20%-$(NH_4)_2SO_4$-saturated PB, then with 5%-$(NH_4)_2SO_4$-saturated PB until the A_{280} of the effluent (typical volume 500 ml) was below 0.05. TH was eluted from the phenyl-Sepharose with a 5 mM-potassium phosphate buffer, pH 7.2; the fractions containing more than 10% of the peak enzymic activity were pooled.

Hydroxyapatite chromatography. If a hydroxyapatite step was included (see the Results section) the pooled fractions from the phenyl-Sepharose column were diluted with 1 vol. of 10 mM-phosphate buffer, pH 6.5, and the pH adjusted to pH 6.5. The solution was loaded on to a 50 ml (1.5 cm × 28 cm) column of hydroxyapatite–Ultrogel equilibrated with 10 mM-phosphate solution, pH 6.5, and washed with 100 ml of the same solution. The enzyme was eluted between 0.1 and 0.15 M-phosphate in a linear gradient (0.01–0.3 M-phosphate) at pH 6.5. The eluate was dialysed overnight against PB.

Heparin-substituted-agarose chromatography. The eluted fractions from phenyl-Sepharose column, containing TH or the dialysed eluate from the hydroxyapatite column, were directly applied on to a 10 ml (1 cm × 13 cm) heparin–Ultrogel column, equilibrated with PB; 30% of column bed had a strong blue–green colour. After washing the column with 100 ml of PB and 50 ml of PB containing 0.1 M-KCl, the pure blue–green TH was eluted with PB containing 0.15 M-KCl, just after a red haem protein absorbing at 412 nm.

Electrophoresis and sucrose-gradient centrifugation

PAGE was performed in the presence of SDS as described by Laemmli (1970), using concentrations of 7.5% and 0.2% (w/v) of acrylamide and bisacrylamide respectively; the gels were stained with silver (Morrissey, 1981). Two-dimensional gel electrophoresis was performed by the method of O'Farrell (1975), with Coomassie Blue R250 staining.

The sedimentation coefficient, *s*, of purified PC12 TH and enzyme present in crude tumour extract by linear sucrose-gradient centrifugation was determined under the conditions described by Musacchio *et al.* (1971), except the use of potassium phosphate buffer instead of Tris/HCl; 50 units of pure PC12 TH or 80 units of TH in a crude extract from PC12 tumours were mixed with proteins of known *s* values and centrifuged (18 h at 27000 rev./

min at 4 °C) in a linear 5–20% (w/v) sucrose gradient; a Beckman SW 27 rotor was used. After centrifugation the gradient was collected and the fractions were analysed for enzymic activities. The *s* values of the reference proteins were: catalase, 11.3 S (Summer & Gralen, 1938); alcohol dehydrogenase: 7.6 S (Theorell & Bonnichsen, 1951); octopine dehydrogenase: 3.4 S (Pho *et al.*, 1970).

Limited proteolysis by chymotrypsin

The method of Cleveland *et al.* (1977) for peptide mapping of protein was used to compare the polypeptide pattern of TH (62 kDa) with its occasional 60 kDa component observed on SDS/PAGE. The two Coomassie Blue-coloured bands were cut out from a first SDS/PAGE gel and soaked separately in 10 ml of 0.125 M-Tris/HCl buffer, pH 6.8, containing 0.1% SDS and 1 mM-EDTA. Then the excised pieces of the first gel were placed in the sample wells of a second SDS/PAGE gel, made of 15% acrylamide and 0.4% bisacrylamide and containing 0.1% SDS. Digestion occurred after addition of 10 ng, 50 ng or 100 ng of chymotrypsin to the sample wells of the second SDS/PAGE gel. After 10 min incubation at 30 °C, electrophoretic migration of the second gel took place for 4 h at 15 mA and then 18 h at 8 mA. The gel was then stained with Coomassie Blue.

Phosphorylation of TH

TH (3–10 mg/ml) was phosphorylated at 30 °C for 30 min in the presence of 1 mM-[γ-^{32}P]ATP and 10 mM-$MgCl_2$ and the catalytic subunit of bovine heart cyclic AMP-dependent protein kinase (0.2 mg of pure subunit/ml), in 50 mM-phosphate buffer, pH 7.2, containing 5 % (v/v) glycerol. The phosphorylation was stopped by cooling the sample on ice, and ATP was subsequently removed by gel filtration on a small Sephadex G-25 column at 4 °C (Andersson *et al.*, 1989*a*). Samples were frozen and stored in liquid N_2 until used. Simultaneously, control incubations were carried out under identical conditions, without ATP, in the presence of kinase. ^{32}P incorporation into TH was measured by using two independent methods (Haavik *et al.*, 1988).

Spectroscopy and determination of the iron and catecholamine content of TH

E.p.r. spectroscopy of TH was performed as described previously with a Bruker ESP 300 system (Andersson *et al.*, 1989*a*) except that 1 mW microwave power was used, with less than 10% saturation of signals at 3.6 K. Resonance Raman spectra were obtained using a spinning quartz cell cooled to 4 °C, with exciting radiation provided by a Spectra Physics model 2030 Ar^+ ion and model 375B dye (Rhodamine 6G) lasers as previously reported (Andersson *et al.*, 1988). Light-absorption spectra were obtained with a Perkin–Elmer 300 u.v.–visible spectrometer. Iron was determined with a Perkin–Elmer model 402 atomic-absorption spectrophotometer with a HGA graphite furnace (Haavik *et al.*, 1988). Catecholamine content of the pure TH was determined as described previously by h.p.l.c. (Andersson *et al.*, 1988) with a Partisphere strong-cation-exchange column and with use of authentic standards.

RESULTS

Purification of rat PC12 TH

The solubility of the rat enzyme was first examined, as the bovine adrenal enzyme is present in a soluble (Haavik *et al.*, 1988) and a membrane-bound form (Kuhn *et al.*, 1990). After homogenization, the total TH activity was determined in the crude tumour extract. This homogenate was then centrifuged, and TH activity was measured in pellets and supernatants. The majority (78%) of the TH activity was recovered in the high-speed supernatant, while a minor fraction was associated with the particulate/membrane fraction.

The soluble fraction of the enzyme was purified by a three-step procedure including $(NH_4)_2SO_4$ precipitation, phenyl-Sepharose chromatography and heparin–Ultrogel chromatography (Table 1). This procedure resulted in a pure enzyme (Fig. 1, lane B). For the resonance Raman experiments (see below), trace amounts of an intensely fluorescent chromophore were removed by including chromatography on a hydroxyapatite–Ultrogel column (50 ml) before the heparin–agarose column. The improvement in purity brought about by the additional hydroxyapatite step was low, as judged by SDS/PAGE (Fig. 1, lane D). The final specific activity of the enzyme was the same with or without this step, whereas the overall yield decreased by about 15%. Standard preparation of pure PC12 TH (phenyl-Sepharose and heparin–Ultrogel) takes $2\frac{1}{2}$ days, whereas inclusion of hydroxyapatite step prolongs the purification by 36 h.

As Table 2 shows, the purified PC12 TH has a high specific activity (520 nmol/min·mg) at pH 7.2, when assayed at low

Table 1. Purification of rat PC12 TH (including the hydroxyapatite step)

TH activity was assayed as described in the Experimental section. The volume of the radioactivity assay mixture was 135 μl and the maximum concentration of protein was 30 μg/ml.

	Activity (units)	Protein (mg)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Homogenate	88500	11800	7	100	1
$(NH_4)_2SO_4$ precipitation (25–50% satn.)	54200	1740	31	61	4.4
Phenyl-Sepharose	44700	420	110	51	15
Hydroxyapatite	28900	110	260	33	37
Heparin–Ultrogel	13700	30	460	15.5	64

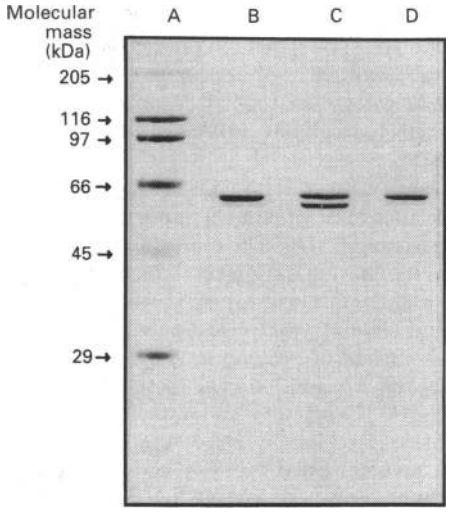

Fig. 1. SDS/PAGE of two preparations of PC12 TH

The gel was silver-stained and 5 μg of pure PC12 TH were applied to each of the lanes B–D. Lane A contained molecular-mass standards. Lane B, PC12 TH stored at −80 °C; lane C, same preparation as in lane B, but stored at −20 °C for 6 months; lane D, preparation with the additional hydroxyapatite step; stored at −80 °C.

Table 2. Comparison of properties of different pterin-dependent hydroxylases

References: [a] the present work; [b] Haavik *et al.* (1988); [c] Andersson *et al.* (1988); [d] Fitzpatrick (1989); [e] Fitzpatrick *et al.* (1990); [f] Haavik *et al.* (1991); [g] Gottschall *et al.* (1982); [h] Bloom *et al.* (1986); [i] Cox *et al.* (1988); [j] Martínez *et al.* (1990*b*); [k] Martínez *et al.*, (1991); [l] See references (*a–l*) for conditions.

Property	Hydroxylase...	Implanted rat PC12 TH[a]	Bovine adrenal TH[a,b,c]	Insect-culture expressed rat TH[d,e]	*E. coli*-expressed human TH-1[f]	Rat (R)[g,h,i,j] and bovine (B)[j,k] liver PAH
Specific activity (nmol/min·mg) at 30 °C with L-Tyr (20 μM) and 6-MePH$_4$ (0.5 mM), pH 7.2 (PC12 and bovine) or pH 7.0 [+Fe(II)]		520	180	1700[l]	1300[l]	–
Specific activity (nmol/min·mg) at 30 °C with L-Tyr (20 μM) and 6-MePH$_4$ (0.5 mM), pH 7.2, +phosphorylation of Ser-40		1120	800	–	–	–
Specific activity (nmol/min·mg) at 30 °C with L-Phe (20 μM) and 6-MePH$_4$ (0.5 mM), pH 7.2 or pH 6.8 and 20 °C(PAH)		52	34	–	–	8000 R[i]/3600 B[i]
Specific activity (nmol/min·mg) at 30 °C with L-Phe (20 μM) and 6-MePH$_4$ (0.5 mM), pH 7.2, +phosphorylation of Ser-40		100	130	–	–	–
Atoms of iron/subunit of enzyme as isolated		0.55	0.7	0.1	0.1	0.94–1.0
Mol of catecholamine/mol of subunit of enzyme as isolated		0.59	0.36	0	0	0
Molecules of catecholamine/atom of iron in enzyme as isolated		1.06	0.55	–	–	–
$\lambda_{max.}$ (nm) and (in parentheses) ϵ (mM-Fe^{-1}·cm^{-1}) of enzyme–catecholate complex		700 (3.1)	700 (1.9)	–	680 (2.0)	700 (1.1*) R
Observed low-temperature e.p.r. *g*-values of catecholamine–Fe(III) complex		7.0,5.2,1.9	7.0,5.2,1.9	–	–	7.0,5.2,1.9
n-Fold activation, pH 7.2 on phosphorylation of Ser-40, under e.p.r. conditions (3 mg of TH/ml, 2 mM-6-MePH$_4$, 1 mM-Tyr, 25 °C)		80–110	8–10	–	–	–

* Only 50% of the iron binds catecholamines[j].

concentrations of enzyme (< 0.03 mg/ml), L-Tyr (25 μM) and the synthetic cofactor 6-MePH$_4$ (0.5 mM). The highly active PC12 TH can also hydroxylate L-Phe to L-Tyr (Table 2), as reported for the bovine enzyme (Fukami *et al.*, 1990). The effect of phosphorylation on enzyme activity measured under the same conditions (i.e. low protein concentration) is also shown.

Characterization of the purified protein

The sedimentation coefficient of TH in crude extracts of phaeochromocytoma tumours and after purification was estimated to be 9.2 S by sucrose-gradient centrifugation (results not shown), corresponding to an apparent molecular mass of 220 kDa for TH in solution. Thus the purified enzyme and TH present in the crude extract both appear to be in a tetrameric form, in agreement with other reports (Kaufman & Kaufman, 1985) and the predicted subunit molecular mass of 55903 Da (Grima *et al.*, 1985).

SDS/PAGE (Fig. 1) shows that PC12 TH migrates as a major band with a molecular mass of 62 kDa and a minor component with molecular mass of 60 kDa. The minor band becomes more pronounced when the enzyme is stored at −20 °C (Fig. 1, lane C). To elucidate the origin of this heterogeneity, we examined the two proteins after mild chymotryptic digestion to determine if the 60 kDa band was a result of proteolysis. The polypeptides with molecular masses of 62 and 60 kDa were isolated from a preparative SDS/PAGE gel and submitted to a second gel electrophoresis after digestion by increasing concentrations of chymotrypsin, as recommended by Cleveland *et al.* (1977). The 62 kDa polypeptide is digested by chymotrypsin, giving two major fragments with molecular masses of 41 and 38 kDa and several smaller fragments. In a similar manner the 60 kDa polypeptide gave rise to a 38 kDa fragment and smaller fragments (results not shown). All the smaller peptides in both cases have equal mobility, thus suggesting the band with molecular mass of 60 kDa is probably a result of a partial proteolysis of the band with molecular mass of 62 kDa. Similar results were also obtained

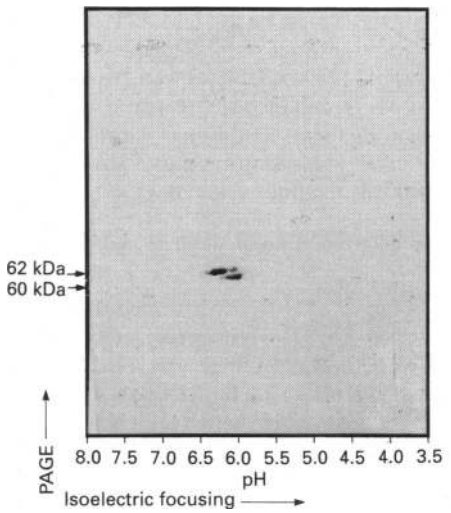

Fig. 2. Two-dimensional gel electrophoresis of purified PC12 TH in the presence of 8 M-urea

A 5 μg portion of pure PC12 TH was analysed by two-dimensional gel electrophoresis by the method of O'Farrell (1975).

in a trypsin-digestion time-course experiment (Bonnefoy *et al.*, 1988). *N*-Terminal sequence analysis of purified PC12 TH shows a maximum of 10% of the PC12 TH truncated after Arg-16 or Arg-36 (J. Thibault, unpublished work).

The electrophoresis of PC12 TH in a polyacrylamide gel in the presence of Ampholines revealed a pI value of 6.2 for PC12 TH (results not shown). By using a two-dimensional electrophoresis-gel technique (O'Farrell, 1975) consisting of isoelectric focusing in the presence of urea, followed by SDS/PAGE, the TH was resolved into five components. Fig. 2 shows the Coomassie Blue-stained gel, and the average isoelectric point was estimated to be

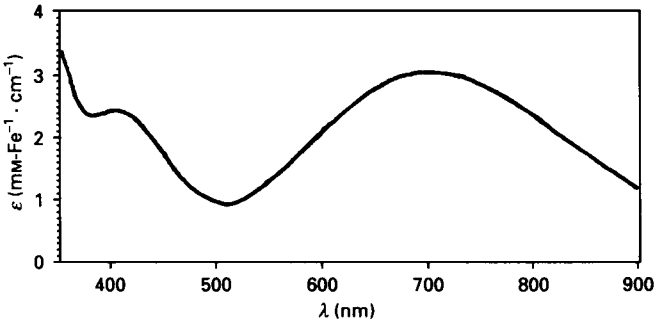

Fig. 3. Visible-light-absorption spectrum of pure rat PC12 TH

The light-absorption spectrum of 10 mg of TH/ml in 0.15 M-KCl/20 mM-phosphate buffer, pH 7.2 (eluate from heparin–Sepharose column) is shown. The light path was 1 cm.

6.3. A similar pattern has been reported for PAH and explained by different amounts of covalently bound phosphate on this phosphoprotein (Smith *et al.*, 1984). Purified bovine TH is also a phosphoprotein, shown by the presence of 0.6 phosphate residue per subunit of bovine TH (Haavik *et al.*, 1988). Thus, by analogy, the presence of five spots of PC12 TH in two-dimensional electrophoresis is rationalized by limited proteolysis of TH, leading to 62 and 60 kDa polypeptides and by the phosphorylation of TH at different amino acid residues.

Light-absorption spectrum and the iron and catecholamine content of purified rat PC12 TH

Pure PC12 TH at 1 mg/ml has an absorbance at 280 nm of 1.0 (cm^{-1}). PC12 TH contains 0.54 ± 0.03 mol of iron per mol of subunit. The isolated PC12 TH has a blue–green colour corresponding to its visible spectrum (Fig. 3), with absorption maxima at 700 nm and 420 nm (ϵ_{700} 3.1 ± 0.2 mM-iron$^{-1} \cdot cm^{-1}$), similar to that of the bovine enzyme. This spectrum is unique for these non-haem iron enzymes as isolated.

Treatment of three different preparations of pure PC12 TH with 8 M-urea or 3% trichloroacetic acid released material with u.v. fluorescence. H.p.l.c. analyses (results not shown) revealed the presence of 0.49 ± 0.09 mol of dopamine and 0.10 ± 0.03 mol of noradrenaline per mol of PC12 TH subunit. The absence of phenylethanolamine *N*-methyltransferase in this rat phaeochromocytoma tumour (Greene & Tischler, 1976) can explain the absence of adrenaline, which is found in the bovine enzyme (Andersson *et al.*, 1988).

Resonance Raman spectrum of PC12 TH as isolated shows the presence of a dopamine–Fe(III) complex

Resonance Raman spectroscopy of metalloproteins gives information regarding the nature of chromophores. The resonance Raman spectrum of PC12 TH, as isolated, with the excitation wavelength at 605 nm (Fig. 4) shows enhanced vibrations at 527, 589, 633, 1272, 1321, 1426 and 1475 cm^{-1}. This spectrum is distinct from those of the metal-tyrosinate proteins (e.g. transferrin, the catechol dioxygenases and purple acid phosphatases), which exhibit tyrosinate vibrations at about 1170, 1270, 1500 and 1600 cm^{-1} (Que, 1983). In contrast, the Raman spectrum of PC12 TH resembles those of the anaerobic complex of protocatechuate 3,4-dioxygenase with catecholate obtained using 647.1 nm excitation and synthetic complexes with bidentate catecholates (Que, 1983; Cox *et al.*, 1988). It is nearly identical with the spectrum of bovine TH (e.g. vibrations at 1272, 1320, 1426, 1476 cm^{-1} (Andersson *et al.*, 1987)); thus the PC12 spectrum has vibration modes typical for an Fe(II)–catecholate complex. The feature near 527 cm^{-1} (Fig. 4) is similar to the chelated catecholate mode observed for $[Fe(catecholate)_3]^{3-}$ at 530 cm^{-1} (Salama *et al.*, 1978), {Fe[bis(salicylidene)ethylenediamine(salen)]catecholate}$^-$ at 529 cm^{-1} (Prytz *et al.*, 1985), and for bovine TH at 530 cm^{-1} (Andersson *et al.*, 1988), suggesting that the catecholate in PC12 TH is co-ordinated in a bidentate

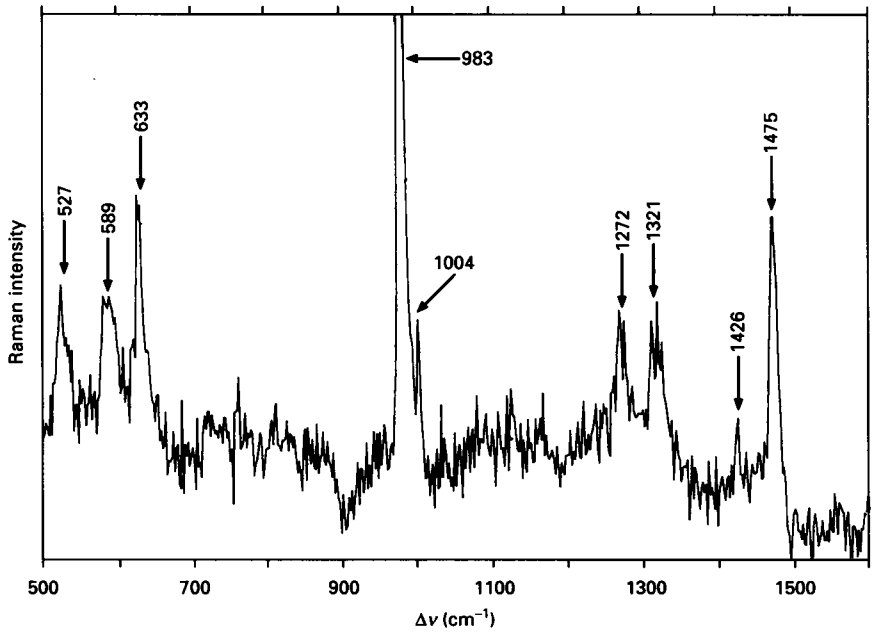

Fig. 4. Resonance Raman spectrum of PC12 TH as isolated

The resonance Raman spectrum of PC12 TH (50 mg/ml) in 0.15 M-KCl/potassium phosphate/50 mM-ammonium sulphate, pH 7.2, at 4 °C in a 100 μl spinning cell is shown. The excitation wavelength was 605 nm (eight accumulations). The features at 983 cm^{-1} and 1004 cm^{-1} are from $(NH_4)_2SO_4$ and phenylalanine respectively. $\Delta\nu$ is Raman (frequency) shift.

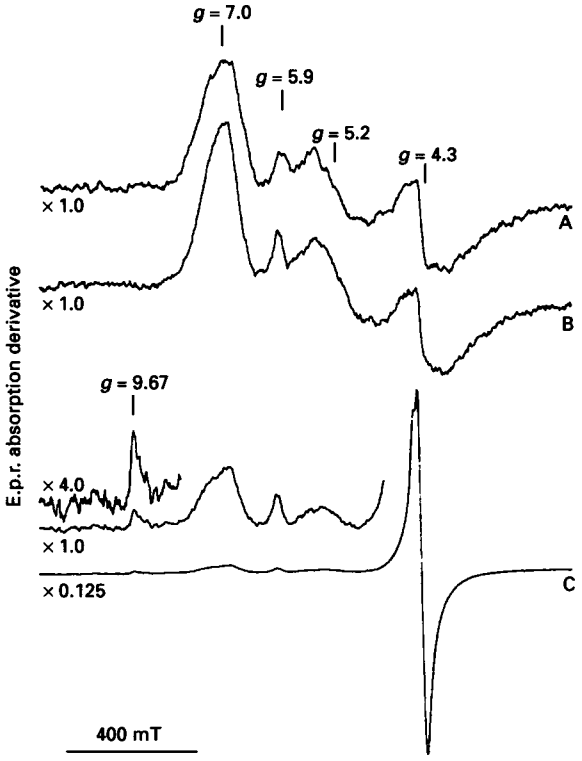

Fig. 5. Low-temperature e.p.r. spectra of phosphorylated bovine and rat PC12 TH

E.p.r. spectra were recorded under the following conditions: 3.6 K; 1 mW microwave power, 10 mT modulation; 9.2 GHz. A, protein kinase A-phosphorylated (0.6 phosphate group per subunit) pure bovine TH (3 mg/ml) in 0.2 M-KCl/0.1 M-potassium phosphate, pH 7.2; two accumulations; B, protein kinase A-phosphorylated (0.6–0.8 phosphate group per subunit) pure rat PC12 TH (3 mg/ml); same buffer as in A, two accumulations; C, sample in B after addition of 1 mM-L-Tyr and incubation for 5 min at 25 °C; four accumulations (spectra scaled to two accumulations and corrected for dilution).

manner as well. Fe(III) model complexes with dopamine {Fe(nitrilotriacetate)dopamine and Fe[*N*-carboxymethyl-*N*-2-picolylglycine(PDA)]dopamine} show Raman spectra similar to those observed for PC12 TH, with vibrations at 1152, 1218, 1266, 1316, 1426 and 1480 cm^{-1} (λ_{ex} = 605–622 nm; results not shown). The PDA complex with adrenaline also exhibits similar vibrations (Andersson *et al.*, 1988; Cox, 1988). The Raman data, together with e.p.r. (see below) and light-absorption spectra, show that the blue–green colour of the isolated PC12 TH is due to a bidentate Fe(III)–catecholamine complex, as observed for the bovine enzyme (Andersson *et al.*, 1988).

E.p.r. spectroscopy of phosphorylated PC12 TH

Low-temperature e.p.r. spectroscopy of metalloproteins is a sensitive probe for changes at the metal centres and gives valuable information regarding the oxidation and spin states of the metal. The blue–green colour of the bovine TH correlates with its Fe(III) e.p.r. spectrum (Haavik *et al.*, 1986, 1988). The e.p.r. spectra of phosphorylated bovine and PC12 THs (Figs. 5*a* and 5*b*, respectively) at 3.6 K and 1 mW have *g*-values of about 7.0, 5.2 and 1.9, and nearly identical linewidths (the e.p.r. spectra of the enzymes as isolated are practically identical with those of the phosphorylated enzymes, except for a 20% loss of intensity upon phosphorylation). Such an e.p.r. spectrum can also be observed for rat PAH and bovine PAH–catecholamine complexes (Bloom *et al.*, 1986; Martínez *et al.*, 1990*a*, 1991). These *g*-values correspond to the calculated *g*-values for the lowest Kramers doublet of high-spin Fe(III) in an environment of axial symmetry with a small rhombic distortion ($|E/D| = 0.04$) (Haavik *et al.*, 1988). The small feature at $g = 5.9$ is from the middle Kramers doublet (Haavik *et al.*, 1988). The e.p.r. spectrum of non-phosphorylated PC12 TH showed only minor changes under turnover conditions or upon the separate addition of substrates or cofactor (results not shown).

When PC12 TH is phosphorylated at Ser-40 (0.6–0.8 mol of phosphate group/subunit) by the catalytic subunit of cyclic AMP-dependent protein kinase, its activity is increased (Table 2). Under the conditions for e.p.r. experiments (Andersson *et al.*, 1989*a*), i.e. 3 mg of TH/ml, 1 mM-L-Tyr and 2 mM-6-$MePH_4$, pH 7.2, the enzymic activity of PC12 TH increased upon phosphorylation by a factor of 80–110 as measured by the formation of dopa at neutral pH. The non-phosphorylated TH with bound catecholamines has very low activity under e.p.r. assay conditions. This low activity is rationalized by the high protein concentration used, i.e. 3 mg of TH/ml, which implies the presence of about 30 μM endogenous catecholamines, a concentration more than one order of magnitude higher than the K_i or half binding constants for catecholamines (Martínez *et al.*, 1990*b*). Under these conditions virtually all iron present is in form of a catecholamine–Fe(III) complex. Thus phosphorylation of TH results in the apparent high activation factor under e.p.r. conditions as compared with the 2-fold increase when PC12 TH is assayed with much lower enzyme, substrate and cofactor concentrations (Table 2). Under e.p.r. assay conditions, the phosphorylated bovine and PC12 TH had similar specific activities. The addition of 1 mM-L-Tyr to the phosphorylated PC12 TH at 25 °C induces a new rhombic species ($|E/D| = 0.33$) with *g* values at 9.7 and 4.3 (Fig. 5*c*). Concomitantly about 67 ± 5% of the axial species vanishes, which corresponds to the degree of phosphorylation of TH (Andersson *et al.*, 1989*a*). Similar substrate effects, not observed for D-Tyr, have been reported for phosphorylated bovine TH (Andersson *et al.*, 1989*a*; Haavik *et al.*, 1989*a*) and rat PAH (Wallick *et al.*, 1982). Further addition of 2 mM-6-$MePH_4$ and incubation at 25 °C for 5 min makes the enzyme iron nearly e.p.r.-silent (results not shown), as observed with the bovine enzyme (Andersson *et al.*, 1989*a*), suggesting that the catecholamine-complexed Fe(III) ion in PC12 TH was reduced to Fe(II) during turnover. Furthermore, the phosphorylated PC12 TH could also be made nearly e.p.r.-silent by incubation with 2 mM-6-$MePH_4$ alone (results not shown), whereas only 10% of the Fe(III) of the non-phosphorylated control enzyme could be reduced to Fe(II) by the cofactor; similar observations have been reported for the bovine TH (Andersson *et al.*, 1989*a*).

DISCUSSION

Purification of rat TH from implanted PC12 cells

Under cell-culture conditions, phaeochromocytoma cells can change a part of their enzyme composition, and Greene & Tischler (1976) used this property to select phaeochromocytoma clones with special characteristics. Goodman *et al.* (1978) also isolated different clones of rat phaeochromocytoma. Starting from a PC12 clone rich in TH, Markey *et al.* (1979) succeeded in purifying and characterizing rat TH from cultured cells. We chose a similar approach in purifying rat TH from tumours of phaeochromocytoma PC12 cells produced after subcutaneous implantation of a few million cells. We obtained 100 g of these TH-rich tumours from ten animals during a 3-week period. By using our purification procedure, it was possible to obtain more

than 30 mg of about 95% pure PC12 TH (the two polypeptides with molecular mass of 62 and 60 kDa together are considered as the pure enzyme). This amount is nearly twice as much as the amount of enzyme that is obtained from the bovine adrenals (which required material from approx. 1000 animals) (Haavik *et al.*, 1988).

Markey *et al.* (1979) found that TH extracted from PC12 cells in culture is a single 62 kDa polypeptide and that no proteolytic processing has occurred, as judged by SDS/PAGE. In the case of our implanted phaeochromocytoma tissue the situation is different; the polypeptide with molecular mass of 62 kDa is partially proteolysed. We do not believe that the proteolysis observed is a function of the purification procedure used, rather the nature of the cells used as the TH source may be responsible for the heterogeneity. As shown in Fig. 1, lane C, our PC12 TH could be partially proteolysed by storage at −20 °C, in spite of the fact that tumours were collected 21 days after implantation in young animals and before any trace of visible necrosis; possibly the tumours could contain higher amounts of proteolytic enzymes compared with PC12 in culture. If the implanted tumours are older than 4 weeks, or if older animals are used, visible necrosis occurs. There are also other differences in TH obtained from cell culture and from tumours. Dix *et al.* (1987) have reported that TH prepared from cultured rat PC12 cells can not hydroxylate Phe to Tyr; however, this activity is observed in our preparations of TH (Table 2), in agreement with Ikeda *et al.* (1967), Kaufman & Kaufman (1985) and rat TH expressed in insect culture (Fitzpatrick, 1991*b*) and findings in bovine adrenal cells in primary culture (Fukami *et al.*, 1990). The ability of TH to hydroxylate L-Phe may be a criterion for a fully active enzyme. It suggests that the implanted PC12 cells behave as in their natural environment, whereas the cells in tissue culture behave differently and result in purified TH with different properties.

Molecular implication for pterin-dependent hydroxylases of the Fe(III)–catecholamine complex and phosphorylation

Table 2 summarizes some properties of pterin-dependent hydroxylases. The rat TH expressed in insect culture contains no endogenous feedback inhibitors and has the highest specific activity reported for the enzyme as isolated when measured in presence of additional Fe(II); similar observations have been made for human TH expressed in *E. coli* (Table 2). When measured at high enzyme concentration, the bovine enzyme is less active because feedback inhibitors co-ordinate about half of the iron present, and PC12 TH has the lowest specific activity because all its iron is co-ordinated by the feedback inhibitors (see the e.p.r. results). Our results clearly demonstrate that the natural highly active rat PC12 TH (Table 2) is similar to bovine adrenal TH, whereas the rat THs purified from cell culture or cloned/expressed THs are different with respect to iron and catecholamine content. Note that rat TH expressed in insect cultures has one phosphate bound per subunit and a very low iron content (Fitzpatrick *et al.*, 1990); phosphorylation favours the Fe(II) form of the TH (see e.p.r. results) and may render iron more weakly bound to the enzyme. The degree of phosphorylation of Ser-40 of TH seems to correlate with an ability to form a new e.p.r.-detectable rhombic species upon addition of the substrate.

The energies of the two catecholate-to-Fe(III) charge-transfer transitions of bovine, human and PC12 THs and the PAH complex are nearly identical (Table 2). Analysis of the energies (wavelength maxima) of the two catecholate-to-Fe(III) charge-transfer transitions can give information about the other ligands to mononuclear Fe(III) (Cox *et al.*, 1988), because these spectra (Fig. 3) are strikingly similar to those of synthetic iron–catecholate complexes both in relative intensity/shape and maxima (Cox *et al.*, 1988). These complexes consequently serve as good models for PC12 TH. A comparison with the data for synthetic adrenaline complexes in methanol (Cox, 1988) and protein complexes suggests that the Fe-binding site in TH consists of three histidine residues and one carboxylate (Cox *et al.*, 1988; Andersson *et al.*, 1988). The proposed iron site, which together with the pterin site constitute the active site, may be similar to those of other aromatic-amino-acid hydroxylases, since three histidine residues (His-317, His-331 and His-336 of the rat TH sequence) are in the highly conserved catalytic domain of these enzymes (Andersson *et al.*, 1988) Furthermore, at least two nitrogen atoms (histidine residues) have also been demonstrated to co-ordinate to the Cu in PAH from *Chromobacterium violaceum* (McCracken *et al.*, 1988). The suggested pterin-binding site for the mammalian hydroxylases in TH residues 310–336 [which correspond to the epitope for a monoclonal anti-idiotype antibody, NS7, against several pterin-utilizing enzymes (Jennings *et al.*, 1991)] is in the same protein domain that we had suggested.

In PC12 TH as isolated the presence of the endogenous catecholamines makes the enzyme nearly inactive when assayed at high enzyme, substrate and cofactor concentrations. The function of the catecholamines associated with TH seems clear; they act as potent feedback inhibitors and, *in vitro*, also protect the enzyme from denaturation (Lazar *et al.*, 1981, 1982; Okuno & Fujisawa, 1991). Catecholamines are also known to be competitive inhibitors with respect to the pterin cofactor (Fitzpatrick, 1988) and they may lower the redox potential of the iron (Andersson *et al.*, 1988). Recent p.m.r. data obtained for rat PAH (Martínez *et al.*, 1991) show that binding of catecholamine to the Fe(III) ion in the enzyme increases the short T_1 value of $^1H^2HO$ from 0.55 s to 0.93 s, demonstrating that when catecholate is not co-ordinated to Fe(III), at least one free Fe(III) site may be accessible to water. This water-accessible site on the iron may represent a putative oxygen-binding site (Andersson *et al.*, 1988). Thus the presence of catechols restricts the access to the enzyme bound Fe(III) and prevents the change of redox state necessary for its catalytic activity.

The hydroxylase activity is strongly inhibited by catechols (Udenfriend *et al.*, 1965; Fitzpatrick, 1988; Martínez *et al.*, 1990*b*, 1991), which suggests that the Fe(III) form takes part in the catalytic cycle (Andersson *et al.*, 1988, 1989*a*); others, however, favour a less important role for the Fe(III) state (Dix & Benkovic, 1988; Fitzpatrick, 1989; Kaufman, 1990). Equilibrium-dialysis and ultrafiltration analysis have demonstrated that catecholamines bind strongly (half binding concentration 0.2–0.5 μM) to the ferric form of both TH and PAH (Martínez *et al.*, 1989, 1990*b*), and it has been shown that the catechols do not bind to the ferrous forms of the enzymes (Martínez *et al.*, 1990*b*). The *E. coli*-expressed human isoforms of TH are strongly inhibited by dopamine (K_i = 0.3–0.5 μM) when assayed in the presence of added Fe(II) (Le Bourdellès *et al.*, 1991), suggesting the generation of Fe(III) during turnover. Analogously, noradrenaline strongly inhibits the phosphorylated insect-expressed rat TH (Fitzpatrick, 1991*a*). The formation of an enzyme-bound Fe(III) species from the pterin-reduced iron has also been observed during enzymic turnover of bovine TH (Andersson *et al.*, 1989*a*). Thus the potential participation of the Fe(III) state in the catalytic cycle of pterin-dependent hyroxylases should not be ignored.

In cultures of PC12 cells (Haycock, 1990) and primary cultures of bovine adrenal cells (Fukami *et al.*, 1990) TH has been shown to be phosphorylated at Ser-40 by protein kinase A. Both bovine and PC12 enzymes are activated by phosphorylation *in vitro*, by decreasing the effect of the feedback inhibition (Ames *et al.*, 1977; Lazar *et al.*, 1982; Okuno & Fujisawa, 1985; Andersson *et al.*, 1989*a*). PAH and TRPH are also effectively inhibited by catechols (DeGraw *et al.*, 1967; Martínez *et al.*, 1991), and

phosphorylation of the rat PAH also lowers the affinity of catecholamines for PAH (Martínez *et al.*, 1990*b*). Phosphorylation increases the off-rate of the bound catecholamine from the hydroxylases (Martínez *et al.*, 1990*b*, Haavik *et al.*, 1990) and thus allows all the iron in PC12 TH to participate in catalysis. Different models for the effects of phosphorylation have been suggested, including the long-range breaking of a salt bridge in TH (Bailey *et al.*, 1989) and phosphorylation at the active site of enzymes (Hurley *et al.*, 1990). We have not detected any magnetic interaction between the Fe(III) and phosphorus (Andersson *et al.*, 1989*a*), as observed between Mo and the covalently bound phosphate in molybdopterin found in, e.g., xanthine oxidase or sulphite oxidase (Rajagopalan, 1991). Short-range electrostatic effects may also be involved in the activation by phosphorylation, as suggested by the decrease of the inhibitory effect of spermine upon protein kinase A phosphorylation of TH (Kiuchi *et al.*, 1987) and similar antagonistic effects between phosphorylation and spermine that have been observed in the mono-oxgenase cytochrome *P*-450 (Mkrtchian & Andersson, 1990) and other hormone-dependent systems (Cohen, 1988).

In conclusion, our results indicate that phosphorylation of Ser-40 of rat PC12 TH reverses the effect of the endogenous feedback inhibitors and suggests that iron-co-ordinated catecholamines have a regulatory role and are of functional importance. Phosphorylation, feedback inhibition and dephosphorylation of TH seem to be elements of a subtle regulatory system for the biosynthesis of catecholamines and may also have significance for the regulation of the aromatic amino acid hydroxylases *in vivo*.

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