Identification of the N-Terminal Heparin Binding Site of Rat Tyrosine Hydroxylase

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Abstract

The first step in catecholamine biosynthesis is the hydroxylation of tyrosine to form dihydroxyphenylalanine (DOPA). This step is rate-limiting and is catalyzed by the enzyme tyrosine hydroxylase. This enzyme binds heparin at some point in the amino terminal domain, the first 155 amino acid residues. In order to further elucidate the binding site of heparin, successive N-terminal truncates were made, and experiments were done to analyze the binding of those truncates to heparin. We found the heparin binding site to be possibly located in the first 85 amino acid residues of the protein.

Introduction

Tyrosine hydroxylase catalyzes the ring hydroxylation of the amino acid tyrosine to form 3,4-L-dihydroxyphenylalanine (L-DOPA) in the catecholamine biosynthetic pathway which yields dopamine, norepinephrine, and epinephrine, as shown by Figure 1 (1). This enzyme is a tetramer, each subunit of approximate molecular weight 56 kD.

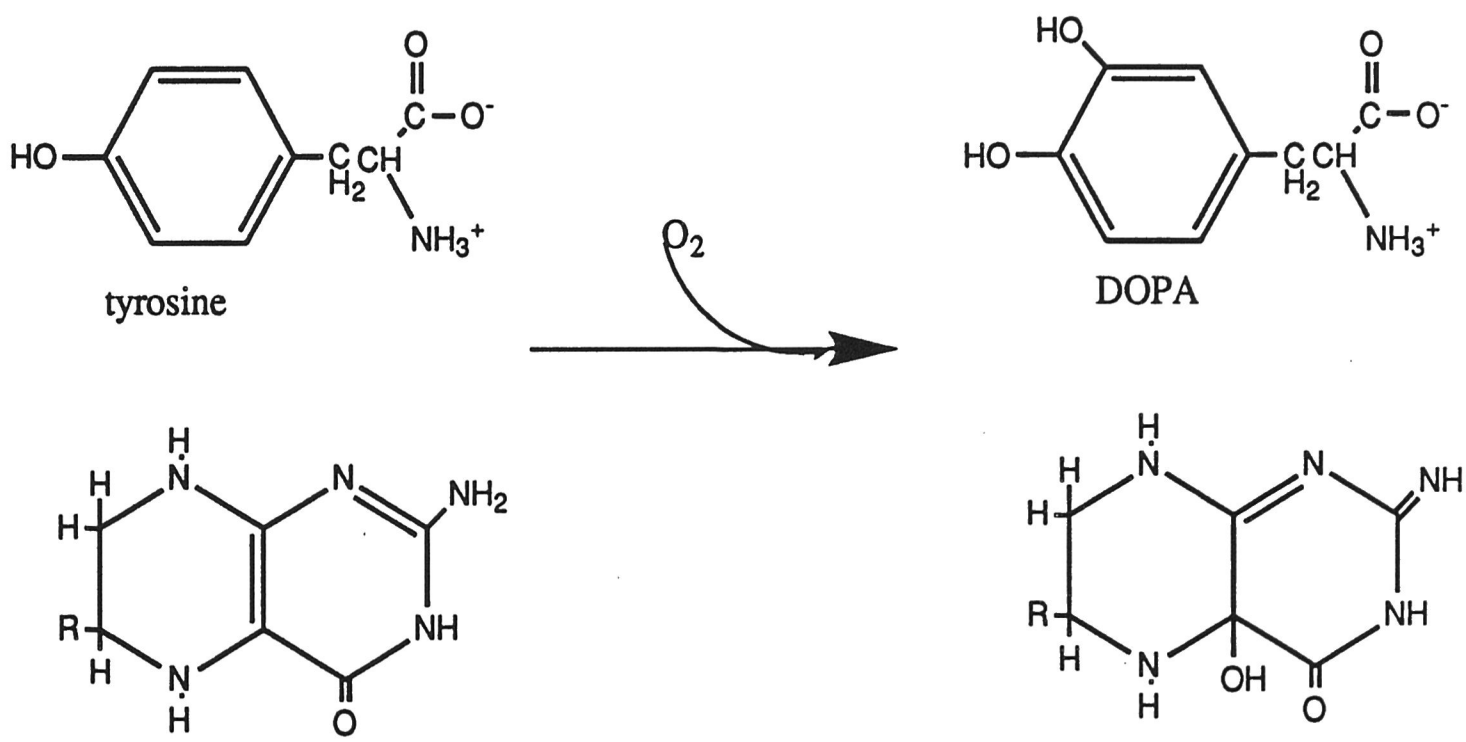


Figure 1. Hydroxylation reaction of tyrosine by tyrosine hydroxylase to form dihydroxyphenylalanine (DOPA) using molecular oxygen and tetrahydropterin (BH_4) as a reducing substrate. Tetrahydropterin is reduced to form 4a-hydroxytetrahydropterin.

This enzyme is a member of a family of enzymes which catalyze ring hydroxylation of aromatic amino acids : tyrosine hydroxylase (TOH), tryptophan

hydroxylase (TRP), and phenylalanine hydroxylase (PAH). All three enzymes utilize molecular oxygen and use tetrahydropterin (BH_4) as a reducing substrate. Also, all three enzymes catalyze the rate limiting step in the biochemical pathways in which they are found: tyrosine hydroxylase in catecholamine biosynthesis (Figure 2), phenylalanine hydroxylase in phenylalanine catabolism (2), and tryptophan in seratonin biosynthesis (3). As the functions of these enzymes are similar, one would expect some degree of homology in the structure of the enzymes. The carboxy-terminal 330 residues (C-term) of the three enzymes are in fact 60% homologous, while the amino-termini (N-term) show little homology (4). Although the functions of the enzymes are similar, the regulatory properties are dissimilar. Tyrosine hydroxylase is activated by heparin, while phenylalanine hydroxylase is not (5). Tyrosine hydroxylase and phenylalanine hydroxylase are both activated by phosphorylation of the N-term, but the number and location of sites are different (2).

Consistent with this, it has been hypothesized that the C-terminal domains of these enzymes are responsible for the catalytic activity of the enzymes, while the N-terminal domains are responsible for regulation. Experiments to confirm this for rat tyrosine hydroxylase were carried out in the laboratory of Dr. Paul F. Fitzpatrick at Texas A&M University. Work was done to express the N-terminal and C-terminal domains separately. This work showed that the C-terminal domain had full catalytic function, but without the expected regulation found in the wild-type enzyme, while the N-terminal domain lacked all catalytic activity, yet could be phosphorylated at the same rate as the wild-type enzyme. In addition, the N-terminal domain could be purified by using the protocol developed for wild-type enzyme, an ammonium sulfate precipitation followed by a heparin-Sepharose affinity column. However, the C-terminal

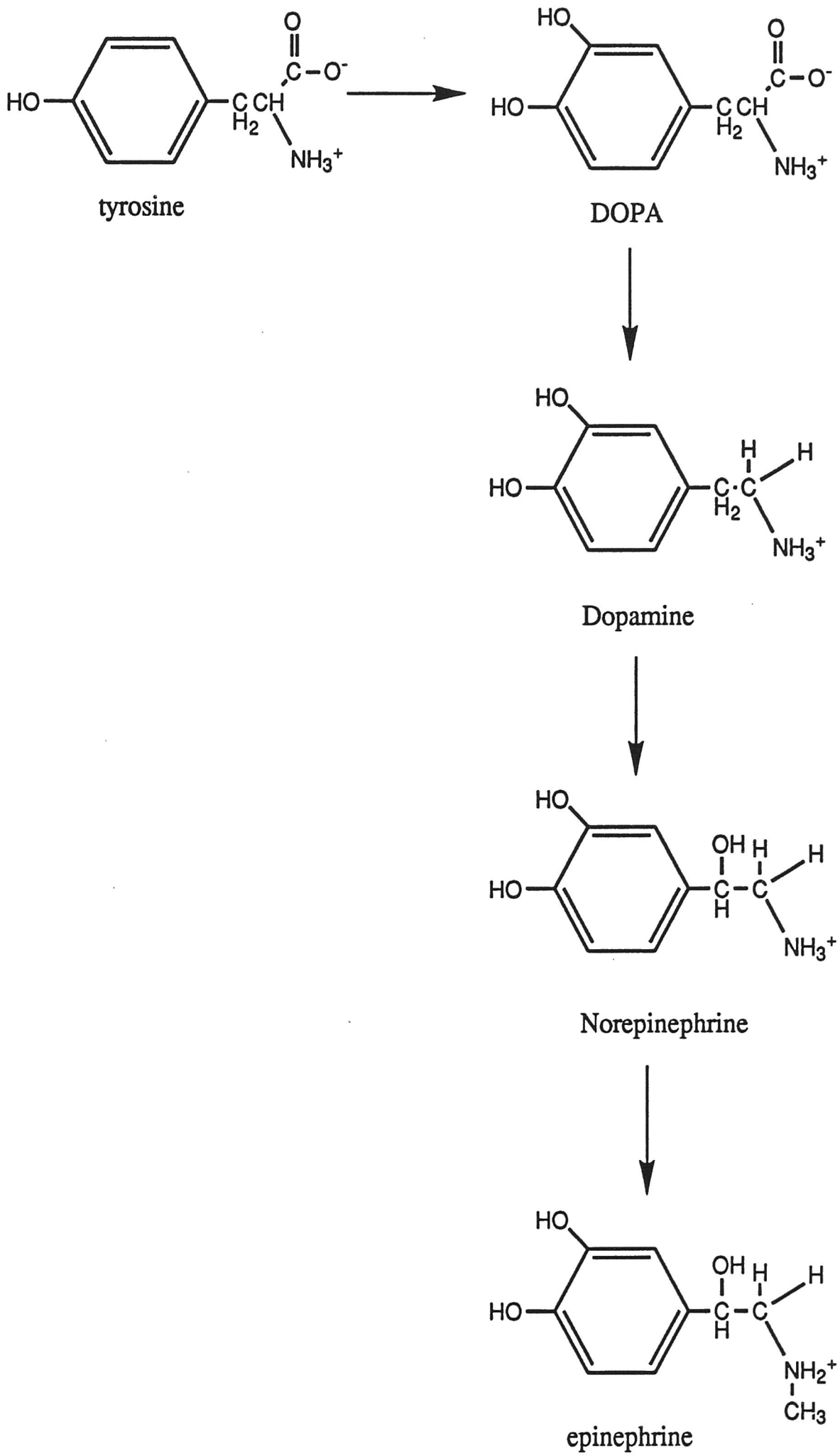


Figure 2. Pathway for the biosynthesis of catecholamine neurotransmitters.

domain did not bind to the heparin column, necessitating the use of an alternative purification protocol. This localized the heparin binding site to the N-terminal 155 amino acids of tyrosine hydroxylase (4).

Heparin is a negatively charged oligosaccharide composed of repeating alternating subunits of α-1,4-L-iduronic acid and D-glucosamine, both of which are sulfated, as shown in Figure 3 (6).

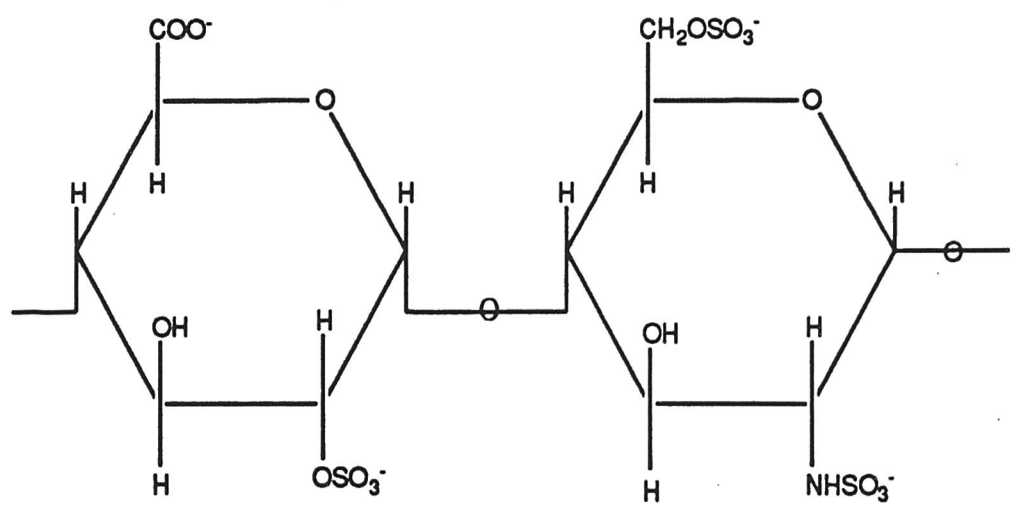


Figure 3. Structure of the disaccharide subunit of heparin.

Heparin binding by proteins is expected to be of an electrostatic nature, as binding can be reversed by the use of a high salt concentration (7). However, electrostatic charges are typically not solely responsible, as other negatively charged polymers usually cannot replace heparin. Instead, a steric fit of heparin in a protein's binding site may be required (8). In a comparison of other proteins which bind heparin, two conserved basic amino acids, most often arginine, in an α-helix 20.4 Å from each other seem to coincide, suggesting either a natural or induced α-helix of protein binds heparin. Thus, the two residues, in opposite directions on the helix, would suggest heparin wrapping around the polypeptide chain, possibly causing a conformational change (9).

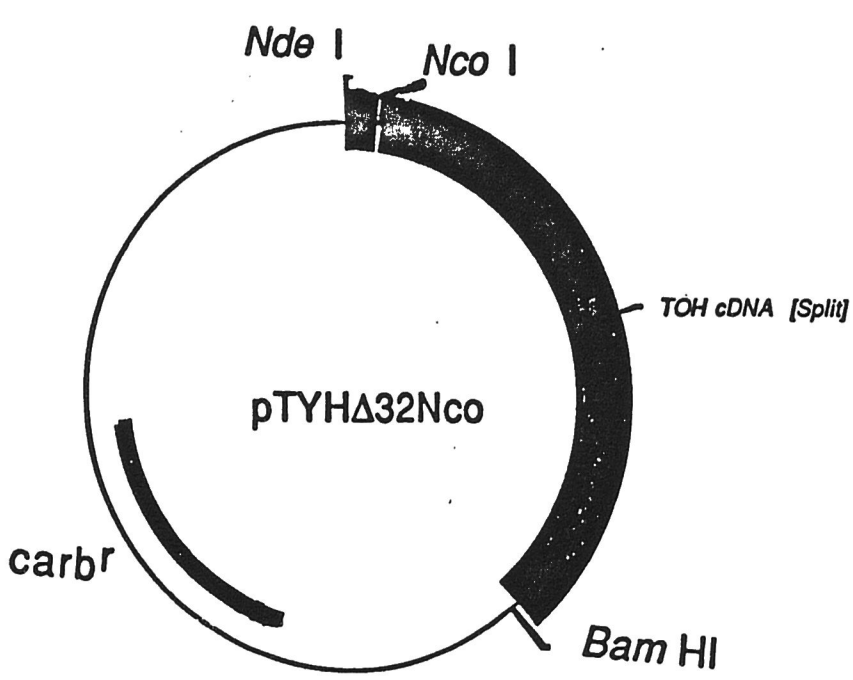
This project focused on attempting to identify a region of amino acids in the N-terminal domain of tyrosine hydroxylase responsible for binding to heparin.

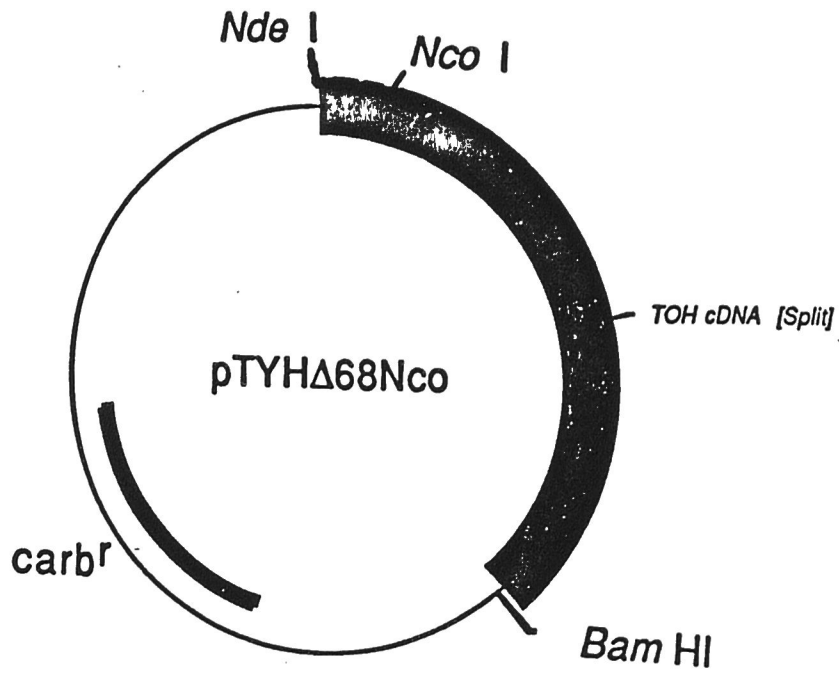
Experimental Procedures

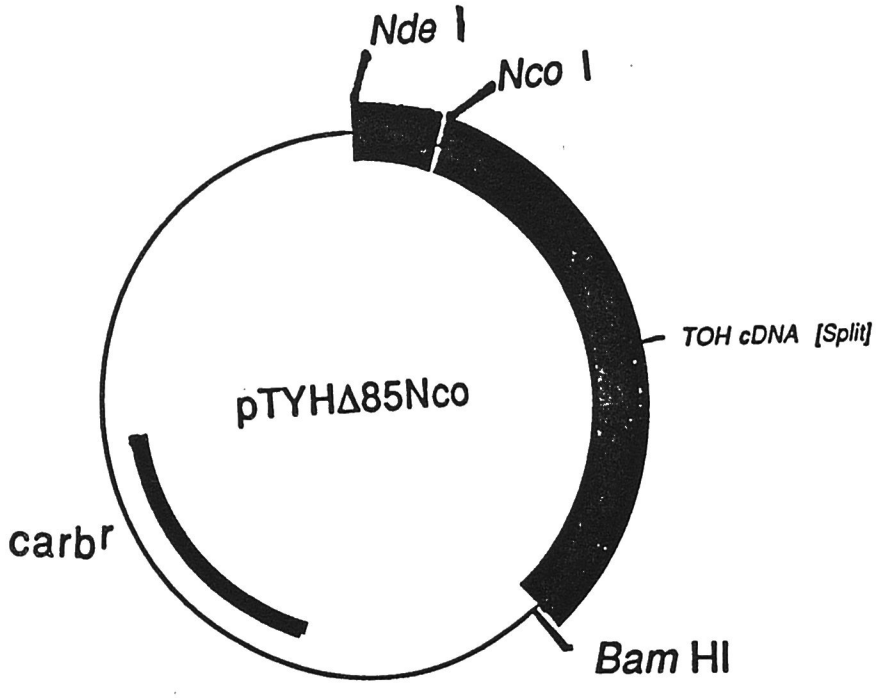
Construction of pTYHΔ mutants

Mutagenesis was carried out using the method of Kunkel et al. (11) on the plasmid pTH6 to create a new *Nde* I site. To construct pTYHΔ32, the oligonucleotide 5'-gtc acg tcc cAT aTg ttc atc gga cgg-3' was used (substituted bases capitalized). pTYHΔ68 was constructed using the oligonucleotide 5'-cct ggg aac cAT atg gag gct gtg-3', pTYHΔ85 was created using the oligonucleotide 5'-aat gct gtt ctc CaT Atg ctc ttc tcc ctg-3', and pTYHΔ120 was created using the oligonucleotide 5'-g cct ggc caT aTg cca ctg gca gg-3'. The mutagenesis mixture was then transformed into competent *E. coli* strain XLI Blue (12) and spread onto plates containing carbenicillin. Possible mutants were then screened for the insertion of the new restriction enzyme site. Experiments to create pTYHΔ32 and pTYHΔ120 were carried out by Dr. S. Colette Daubner.

In addition, new *Nco* I sites were generated in pTH6. These were placed in the same approximate locations as the *Nde* I mutants, as shown in Figure 4, and designated pTYHΔ__Nco. Oligonugleotides used for these mutageneses were: for pTYHΔ32Nco, 5'-gtc acg tcc ccC aTg Gtc atc gga cgg-3'; for pTYHΔ68Nco, 5'-cct ggg aac ccC Atg gag gct gtg-3'; for pTYHΔ85Nco, 5'-gct gtt ctc aCc Atg Gtc ttc tcc ctg-3'; and for pTYHΔ128Nco, 5'-gga agc ccc cCc Atg gag tat ttt gtg-3'. Experiments to create pTYHΔ128Nco were carried out by Dr. S. Colette Daubner.







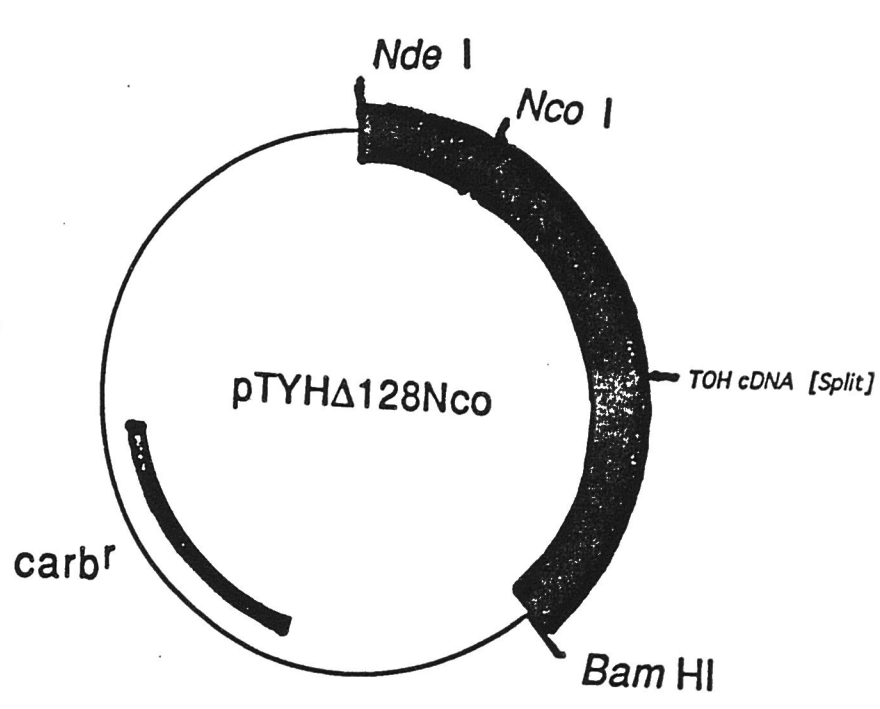


Figure 4. Mutant pTYHΔ__Nco plasmids, showing the approximate positions of the new *Nco* I sites.

Construction of pETOHΔ mutants

The *Nco* I to *Bam* HI fragments were excised and cloned into pET11d of Studier et al. (13). The resulting plasmids, which also conferred carbenicillin resistance, contained the truncated tyrosine hydroxylase gene downstream from a T7 RNA polymerase promoter. These plasmids were then transformed into competent *E. coli* strain BL21DE3 (14). Experiments to construct pETOHΔ68, pETOHΔ85, and pETOHΔ128 were performed by Dr. S. Colette Daubner. pETOHΔ32 was not constructed.

Expression of N-terminal truncates

BL21DE3 pETOHΔ85, BL21DE3 pETOHΔ128, and BL21DE3 pETOHΔ155 37kD were plated on Luria-Bertani medium with agar containing 100 μg/ml carbenicillin and grown at 37 °C for approximately twelve hours. A single colony was then transferred to five milliliters of Luria-Bertani medium containing 100 μg/ml carbenicillin (LB-carb) and grown for twelve hours at 37°C shaking at 300 rpm. Two hundred milliliters (200 ml) of LB-carb was then inoculated using two milliliters (2 ml) of the overnight culture. These two hundred milliliter cultures were then grown for approximately two hours, until they reached an A_{600} of 0.6-0.8 (mid-log phase). Aliquots of each culture were then taken to analyze induction. The cultures were then induced with IPTG to a final concentration of 0.5 mM. Induced cultures were then grown for two hours. Cells were centrifuged at 4,000 RPM at 4 °C for ten minutes in an SS34 rotor in a Sorvall RC-5B centrifuge. The supernatants were removed, and the cells were frozen overnight.

Analysis for Heparin Binding

Frozen cell pellets for BL21DE3 pETOHΔ85, BL21DE3 pETOHΔ128, and BL21ΔE3 pETOHΔ155 37kD were weighed and suspended in six milliliters homogenization buffer (50 mM Tris-Cl, 1 mM EDTA , 10% glycerol, 100 µg/ml phenylmethylsulfonyl fluoride (PMSF), 1 µM leupeptin, and 1 µM pepstatin, pH 7.1,) per gram of frozen cells. Lysozyme was added to a final concentration of 50 µg/ml, and the cells were incubated on ice for twenty minutes. They were then sonicated on setting 1, 10% duty for 4 cycles of 5 second bursts with 20 second rests on a Branson Sonifier 450. The lysates were then spun for fifteen minutes at 15,000 rpm at 4 °C in an SS34 rotor in a Sorvall RC-5B centrifuge. The supernatants were saved; the pellets were discarded.

Columns of heparin-Sepharose were prepared and washed with homogenization buffer containing 2M KCl (high salt buffer). One milliliter (1 ml) samples of the 15K supers were loaded onto the columns, and "filtrate 1" was collected. The column was then washed twice with one milliliter aliquots of homogenization buffer, and "filtrate 2" and "filtrate wash" were collected. Three one milliliter aliquots of high salt buffer were added to the columns, and "eluate 1," "eluate 2," and "eluate wash" were collected.

Samples of the 15K super, filtrate 1, filtrate 2, eluate 1, and eluate 2 for each TOHΔ85, TOHΔ128, and TOHΔ155 37 kD were then run on a denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15). Tyrosine hydroxylase activity assays were done by following the release of ^{3}H from 3,5-[^{3}H] tyrosine during the hydroxylation reaction (16).

Results

To localize the heparin binding site, N-terminal truncates were produced (Figure 5). To do this, site-directed mutagenesis was performed on pTH6 to introduce a new restriction enzyme site at the point of truncation in the gene. The shortened gene was then excised and cloned into an expression vector. Cell cultures containing the new expression vector with the shortened gene were grown, induced, and lysed.

Figure 5. Primary sequence of rat tyrosine hydroxylase, showing positions of truncations. Conserved amino acids are in bold type.

The plasmid pTH6, as constructed previously, contains the cDNA for rat tyrosine hydroxylase inserted between a *Nde* I site at the beginning of the wild-type gene and a *Bam* HI site at the end of the gene. The plasmid also confers resistance to the antibiotic carbenicillin (10). Site directed mutagenesis was first carried out on pTH6 to generate new *Nde* I sites at the truncation sites, using oligonucleotides synthesized by the Texas A&M Gene Technologies Laboratory.

However, problems arose in the ability to obtain consistent batches of *Nde* I. Supplies from New England Biolabs, Boehringer Mannheim, United States Biochemical, and Promega all proved to work only on a limited basis. Therefore, additional mutagenesis was carried out to introduce *Nco* I sites into the N-terminus.

Expression of TOH Δ mutants

Expression was carried out using *E. coli* strain BL21DE3. BL21 lacks the proteases *lon* and *ompT* such that it can be used for the overexpression of heterologous proteins. The DE3 lysogen is inserted into the bacterial genome and is a λ bacteriophage derivative containing the gene for T7 RNA polymerase under control of the *lac* operon, such that significant levels of T7 RNA polymerase will be produced only upon induction by lactose or a non-metabolizable analog, isopropyl β-D-thioglucopyranoside (IPTG) (14). Thus, upon induction by IPTG, T7 RNA polymerase is produced, and transcription and translation of the truncated tyrosine hydroxylase gene occurs.

Induction was tested using the aliquots removed just prior to induction of the larger culture. 50 µl were removed into 500 µl of LB, and 15 µl was spread onto each of two plates: LB-carb and LB-carb-IPTG (containing 0.5 mM IPTG). These plates were then grown overnight. If induction takes place in all of the

cells, then the cells will not grow, and colonies will not be visible after incubation at 37 °C. However, if induction does not occur, and T7 RNA polymerase and our protein of interest is not produced, then the cells will grow and colonies will be visible after incubation at 37 °C. On all plates grown on LB-carb, a lawn of cells was seen. On the LB-carb-IPTG plates, growth was dramatically reduced: for pETOHΔ85 BL21DE, I saw six colonies, for pETOHΔ128 BL21DE, I saw three colonies, and for pETOHΔ155 37 kD BL21DE, I saw approximately two hundred colonies. However, for pETOHΔ155 BL21DE, two hundred colonies are typically seen for preps yielding large amounts of the protein. This would seem to indicate that large amounts of the mRNA for my protein of interest were generated.

The cell lysates had little activity. Wild-type tyrosine hydroxylase from a separate prep yielded 177 u/ml, where one unit is defined as 1 nmole tyrosine hydroxylated/minute. TOHΔ155 37kD for my experiments yielded 201 u/ml for the 15K super, 51-76 u/ml for filtrates 1 and 2, and 1.4-1.9 u/ml for eluates 1 and 2. Activities for TOHΔ85 and TOHΔ128 were much lower. TOHΔ85 yielded 4.0 u/ml for 15K super, 1.3-4.2 u/ml for filtrates 1 and 2, and 1.5-1.6 u/ml for eluates 1 and 2. TOHΔ128 yielded 1.4 u/ml for 15K super, 1.4-5.0 u/ml for filtrates 1 and 2, and 0-1.9 u/ml for eluates 1 and 2. These activities are very low, possibly indicating that neither binds to heparin.

Heparin Binding

That activity was found in the filtrates for TOHΔ155 37kD indicates that this protein does not bind heparin. Although activity is minimal, the activity found in TOHΔ85 and TOHΔ128 would seem to indicate that these do not bind heparin, either.

Discussion

Little is known about the structure of the N-terminus of tyrosine hydroxylase (4). It is known that the N-term of the protein does bind to the oligosaccharide heparin. In the work described in this, very little activity could be detected for the protein expressed. However, it was determined that the TOHΔ155 37kD mutant was fully active, and confirmed that the binding site for heparin is in the first 155 residues, as this protein did not bind heparin. Also, it is possible to speculate that the binding site for heparin is within the first 85 amino acid residues, as what activity could be found for TOHΔ85 and TOHΔ128 was found in the filtrates, not in the eluates.

The probable cause for the lack of activity is poor cell lysis. The induction experiment showed that the cells did produce the mRNA for my genes of interest. However, as the cell culture size was small, extra care was taken in the lysis step in order to keep from denaturing all proteins. Perhaps this "extra care" prevented the recovery of more truncated tyrosine hydroxylase.

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