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FOR THE RECORD

Deletion mutants of tyrosine hydroxylase identify a region critical for heparin binding

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Abstract: Phenylalanine hydroxylase, tyrosine hydroxylase, and tryptophan hydroxylase constitute a family of tetrahydropterin-dependent aromatic amino acid hydroxylases. It has been proposed that each hydroxylase is composed of a conserved C-terminal catalytic domain and an unrelated N-terminal regulatory domain. Of the three, only tyrosine hydroxylase is activated by heparin and binds to heparin-Sepharose. A series of N-terminal deletion mutants of tyrosine hydroxylase has been expressed in *Escherichia coli* to identify the heparin-binding site. The mutants lacking the first 32 or 68 amino acids bind to heparin-Sepharose. The mutant lacking 76 amino acids binds somewhat to heparin-Sepharose and the proteins lacking 88 or 128 do not bind at all. Therefore, an important segment of the heparin-binding site must be composed of the region from residues 76 to 90. All of the deletion mutants are active, and the Michaelis constants for pterins and tyrosine are similar among all the mutant and wild-type enzymes.

Keywords: catecholamine biosynthesis; domains; kinetics; mutagenesis; phenylalanine hydroxylase; tetrahydrobiopterin; tyrosine hydroxylase

Tyrosine hydroxylase (TH) catalyzes the conversion of tyrosine to L-dihydroxyphenylalanine, the first and the rate-limiting step in the pathway that yields the catecholamine neurotransmitters (Weiner, 1979; Zigmond et al., 1989). The enzyme is a tetramer of identical subunits of 498 amino acid residues (Grima et al., 1985). TH displays functional and structural similarities to phenylalanine hydroxylase and tryptophan hydroxylase (Kaufman & Fisher, 1974). All three contain iron, catalyze ring hydroxylation of aromatic amino acids, and utilize tetrahydrobiopterin (BH_4) as a substrate. All are regulated by phosphorylation at serines in their N-termini. All are rate-limiting catalysts for important metabolic pathways. It has been suggested that all three consist of a catalytic domain (C domain) that is located at the C-terminal end of the protein and a regulatory N-terminal domain (R domain) (Abate & Joh, 1991). A tetramerization moiety exists at the very C-terminal end of the enzymes (Lohse & Fitzpatrick, 1993). The amino acid sequences of the three are 75% identical in their 330 C-terminal residues, but their N-termini display little similarity (Grenett et al., 1987).

Brown et al. (1987) have suggested that the R domains arose from genes that were recruited from different sources to combine with the common gene for the catalytic core. This implies that, during the process of constructing a series of finely tuned amino acid hydroxylases, evolution resulted in three R domains conferring upon the same C domain the unique regulatory properties of each enzyme. Because these enzymes are rate-limiting for important metabolic pathways, they are exquisitely regulated. For TH, the major modes of regulation include feedback inhibition by metabolites, relief of feedback inhibition by phosphorylation (Daubner et al., 1992), and activation by anions (Vigny & Henry, 1981). The structure of the R domain and its ability to confer upon the prototypic hydroxylase the capacity to be regulated by these mechanisms are of great interest.

One difference among the three enzymes that is a direct result of the existence of different R domains is that TH binds to polyanions such as polyglutamate, phosphatidylserine, and heparin (Katz et al., 1976). We recently constructed a truncated form of TH using DNA recombinant techniques; it lacks the amino-terminal 155 residues and does not bind to heparin (Daubner et al., 1993). In order to study in more detail the anion-binding site of the R domain of TH, we have now constructed a series of deletion mutants of TH, each containing progressively less of the R domain. This paper describes their construction, expression, catalytic activities, and heparin-binding activities.

The region of homology between the three BH_4-dependent hydroxylases begins at valine 164 of TH (Fig. 1). To generate mutants containing portions of the R domain, the first 32, 68, 76, 85, 88, 96, and 128 amino acids were deleted by introducing new start codons into the pET vector containing the cDNA for rat TH. (The mutant missing the first 32 residues will be referred to as Δ32TH, the first 68, Δ68TH, etc.) Due to the use of *Nco* I restriction sites (CCATGG) to enter new start codons, some amino acid substitutions at the N-termini resulted, several leading to a change in charge properties. These are recorded in Figure 1.

The pET vector expression system (Novagen) utilizes isopropyl β-D-thioglucanopyranoside (IPTG)-inducible T7 RNA poly-

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        10         20         30         40         50         60
MPTPSAPSPQ PKGFRRAVSE QDAKQAEAVT SPRFIGRRQS LIEDARKERE AAAAAAAAAV
                                 Δ32TH: MVIGRRQS LIEDARKERE AAAAAAAAAV

        70         80         90        100        110        120
ASSEPGNPLE AVVFEERDGN AVLNLLFSLR GTKPSSLSRA VKVFETFEAK IHHLETRPAQ
Δ68TH: ME AVVFEERDGN AVLNLLFSLR GTKPSSLSRA VKVFETFEAK IHHLETRPAQ
      Δ76TH: MDGN AVLNLLFSLR GTKPSSLSRA VKVFETFEAK IHHLETRPAQ
           Δ85TH: MVFSLR GTKPSSLSRA VKVFETFEAK IHHLETRPAQ
         Δ88TH: MG GTKPSSLSRA VKVFETFEAK IHHLETRPAQ
              Δ96TH: MARA VKVFETFEAK IHHLETRPAQ

       130        140        150        160        170        180
RPLAGSPHLE YFVRFEVPSG DLAALLSSVR RVSDDVRSAR EDKVPWFPRK VSELDKCHHL
Δ128TH: ME YFVRFEVPSG DLAALLSSVR RVSDDVRSAR EDKVPWFPRK VSELDKCHHL
                      Δ155TH: MRSAR EDKVPWFPRK VSELDKCHHL
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Fig. 1. Amino acid sequences of the R domains of wild-type rat TH and N-terminal deletion mutants. Names of deletion mutants and any residues at the N-termini of the mutants that differ from wild-type enzyme are underlined. The oligonucleotides used for introducing new start codons were as follows:

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Δ32   gtc acg tcc ccc atg gtc atc gga cgg
Δ68   cct ggg aac ccc atg gag gct gtg
Δ76   gtg gta ttt gag gcc atg gat ggg aat
Δ84   gct gtt ctc acc atg gtc ttc tcc ctg agg
Δ88     g ctc ttc tcc ccc atg ggt aca aaa ccc
Δ96     a ccc tcc tcc atg gct cgg gct gt
Δ128  gga agc ccc ccc atg gag tat ttt gtg
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Start codons are in bold, and *Nco* I sites are underlined.

merase (Studier et al., 1990). Expression levels vary due to many factors. We found large differences in expression efficiency after making deletions of the R domain of TH. The activity values for lysate supernatants, after optimization of growth and sonication conditions, were: wild-type TH, 0.15 units (μmol product per min at 30 °C, pH 7.0)/mL; Δ32TH, 0.344 units/mL; Δ68TH, 0.283 units/mL; Δ76TH, 0.068 units/mL; Δ85TH, 0.012 units/mL; Δ88TH, 0.093 units/mL; Δ96TH, 0.011 units/mL; 128TH, 0.242 units/mL; and Δ155TH, 0.066 units/mL. As judged by the intensity of bands on denaturing polyacrylamide gels after electrophoresis (SDS-PAGE) (Laemmli, 1970) of supernatants and pellets, the mutants Δ32TH, Δ68TH, Δ76TH, and Δ155TH were produced by *E. coli* in totally soluble forms because no protein of the correct molecular weights appeared in the pellets. Thus, differences in activity must have been due to different levels of expression (V_{max} parameters for purified Δ32TH, Δ68TH, and Δ155TH appear in Table 1 and were comparable). Mutants Δ85TH, Δ88TH, Δ96TH, and Δ128TH were expressed in such overabundance they aggregated and were very difficult to solubilize; they were detected after separation of pellet samples on SDS-PAGE. If we found all of the protein produced at 37 °C in aggregates, we repeated the growth at 30 °C and induced with lactose instead of IPTG. If the target protein was still insoluble, expression was attempted including the lysozyme-coding plasmids pLysS or pLysE. These modifications were made in order to slow down the overproduction of protein, and in several cases this strategy worked. For mutant Δ88TH the lower temperature and utilization of pLysE enabled us to isolate enough protein to study; for mutant Δ128TH these conditions almost eliminated aggregate formation. In contrast, useful quantities of soluble Δ85TH and Δ96TH were never obtained so we could not study the effect of those deletions. It is interesting that only mutants with deletions in the residue 85–128 range gave this problem.

The binding of the R domain of TH to polyanions and anions results in enzyme activation, which may be a major factor in the regulation of enzyme activity. The polyanion most often used is heparin. We determined heparin binding by performing chromatography with heparin-Sepharose resin (Pharmacia). Approximately equal amounts of each mutant were applied to heparin columns under equivalent conditions of ionic strength; Table 1 shows the results. Wild-type TH bound completely to the resin and eluted upon application of a KCl gradient, with no loss of activity. Mutants Δ32TH and Δ68TH also bound to the resin and eluted with a KCl gradient. Approximately 44% of mutant Δ76TH bound to the resin. The bound fraction eluted upon application of the KCl gradient, and overall recovery was 100%. Mutants Δ32TH, Δ68TH, and Δ76TH eluted from heparin-Sepharose at approximately the same KCl concentration at which wild-type TH elutes, about 0.35 M. Mutants Δ88TH and Δ128TH did not bind to heparin and were completely recovered in the column filtrates. Application of a KCl gradient did not remove any catalytic activity or any protein with the MW of these last two mutants, as ascertained by denaturing polyacrylamide gels.

Because deletions of the first 68 amino acids did not change the affinity of the enzyme for heparin-Sepharose, it is clear that this region is not necessary for heparin binding. Deletion of 76 amino acids did decrease the enzyme's ability to bind to heparin-Sepharose, and removal of 88 amino acids abolished it entirely.

Table 1. *Properties of N-terminal deletion mutants of tyrosine hydroxylase*

Protein	MW	Inclusion bodies[a]	Bind to column?[b]	Units in column filtrate[c]	Units in column eluate	$K_{M6\text{-}MPH_4}$[d]	K_{MBH_4}[e]	K_{tyr}[f]	V_{max}[g]
Wild type	55.9K	No	Yes	0	2.7	53 ± 31	18 ± 8	30 ± 7	197 ± 19
Δ32TH	52.5K	No	Yes	1.1	4.0	30 ± 6	16 ± 2	31 ± 4	172 ± 10
Δ68TH	48.9K	No	Yes	0.2	2.9	23 ± 6	8 ± 2	25 ± 6	135 ± 10
Δ76TH	47.9K	No	Yes	1.5	1.2	17 ± 6	10 ± 1	25 ± 10	
Δ85TH	47.1K	Yes							
Δ88TH	46.6K	Yes	No	2.8	0	36 ± 14	20 ± 1	45 ± 14	
Δ96TH	45.8K	Yes							
Δ128TH	42.2K	Yes	No	3.0	0.2	25 ± 4	10 ± 1	23 ± 3	
Δ155TH[h]	39.0K	No	No			21 ± 4	7 ± 3		221 ± 20

[a] Refers to whether protein was insoluble when expression was carried out at 37 °C or 30 °C. Sonication to release enzyme from cells was performed using six bursts of 30 s at 45 W with 2-min intervals.
[b] Approximately 3.00 units (μmol P/min/mL) of each mutant, in 0.15 M ammonium sulfate, were applied to 9 mL heparin-Sepharose.
[c] Percent units recovered in filtrate, washes, plus eluate was routinely >90%.
[d] Michaelis constant for 6-methyltetrahydropterin was determined as previously described (Daubner et al., 1992).
[e] Michaelis constant for tetrahydrobiopterin was determined as previously described (Daubner et al., 1992).
[f] Michaelis constant for tyrosine was determined using 6-methyltetrahydropterin as the pterin substrate.
[g] V_{max} value was determined only for proteins purified to homogeneity.
[h] Data for Δ155TH were determined and reported previously (Daubner et al., 1993).

The amino acid sequence of the R domain of TH contains amino acid residues that might contribute to a polyanion-binding region; 10 positive charges (lysines and arginines) lie between lysine 12 and arginine 49. However, this region is clearly not responsible because Δ68TH binds to heparin-Sepharose. The next region of positive charge density lies between arginine 77 and arginine 121, where there are eight basic residues. Mutant Δ76TH is missing arginine 77, and mutant Δ88TH is missing arginine 77 and arginine 90. These two arginine residues, properly situated, appear to be critical for the binding of polyanions. This spacing (the two arginine residues are not a multiple of four positions apart) is not consistent with them being presented to the ligand along the same side of an α-helix. Furthermore, the CD spectrum of the R domain demonstrates that it consists mostly of β structure (Daubner et al., 1993). With the data at hand it is reasonable to assume that the arginine residues are part of a binding site that is made up of more than one separate structural element.

Steady-state kinetic analyses were performed on wild-type TH and the deletion mutants. Michaelis constants and V_{max} values appear in Table 1. We could find no significant differences among the proteins in K_M values for BH_4, 6-methyltetrahydropterin, or tyrosine. All the mutants were inhibited by tyrosine concentrations above 30 μM, as is wild-type TH, when assayed with BH_4. We determined V_{max} values for the proteins that were purified on heparin-Sepharose. All the V_{max} values are comparable. The data clearly show that the R domain has no intrinsic effect on these kinetic parameters. Due to the fairly recent cloning of TH into *E. coli* vectors, most previous work done on TH either activated by cleavage with trypsin or phosphorylation with cAMP-dependent protein kinase was performed on enzyme produced in pheochromocytoma cells or nervous tissue. Enzyme produced in these tissues is isolated with bound catecholamine, and we have shown that the major mode of activation by phosphorylation of TH is the alleviation of catecholamine inhibition (Daubner et al., 1992). Enzyme produced in *E. coli* is isolated without bound catecholamine, so we are studying kinetic parameters intrinsic to the protein. Our data on the Δ155TH mutant show that removal of the entire R domain does not alter these intrinsic kinetic parameters (Daubner et al., 1993).

The inability of proteins to fold properly during expression leads to their precipitation into aggregates termed inclusion bodies. We found that inability of TH to fold properly is coincident with the loss of heparin binding. Deletion mutants Δ85TH, Δ88TH, and Δ96TH formed the largest quantities of insoluble, wrongly folded protein, and the deletion of the first 88 amino acids caused TH to lose affinity for heparin-Sepharose. Clearly something drastic has happened to the protein structure upon deletion of the first 85 amino acids. Because Δ76TH did not overexpress well (0.068 units/mL as compared to 0.344 for Δ32), nor bind well to heparin-Sepharose (never did as much as 50% stick to the resin), we see this position as a region whose deletion causes the same but less drastic changes to the protein structure. We suggest that TH does not fold correctly unless its first 76–85 amino acids are present as a guide to folding (or unless deletion is more drastic because mutant Δ155TH does not form insoluble aggregates). The region from residues 1 to 68 serves as the phosphorylation subdomain (protein kinases modify serines 19, 31, and 40 [Campbell et al., 1986]), and the region from 1 to 85 may be a subdomain that helps to guide the proper folding of the rest of the protein. The region from residues 68 to 90 is critical for anchoring a polyanion such as heparin. The region of the R domain from residues 90 to 155 may be a second subdomain, which constitutes the remainder of the anion binding site. It is doubtful that such a large polyanion as heparin is bound merely by two arginine residues, and the region from arginine 77 to arginine 121 contains a cluster of eight basic residues, which may add to the binding capacity after anchoring has occurred at arginines 77 and 90.

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