Limited Proteolysis of Tyrosine Hydroxylase by Ca^{2+}-Activated Neutral Protease (Calpain)†

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ABSTRACT: Limited proteolysis of tyrosine hydroxylase (TH) by calpain, Ca^{2+}-activated neutral protease, was studied. Cleavage of TH with calpain in vitro produced molecules having M_rs of approximately 57 000 and 56 000 in SDS–polyacrylamide gel electrophoresis. The N-terminal amino acid sequence, Ser-Pro-Arg-Phe-Val, of the 56-kDa species indicated that calpain cleaved off the N-terminal region (residues 1–30) encoded by the first exon including Ser-8 and Ser-19, the phosphorylation sites by proline-directed protein kinase (PDPK) and by Ca^{2+}/calmodulin-dependent protein kinase II (kinase II), respectively, from the native TH. The removal of the N-terminal region from the native molecule induced a slight but significant activation of TH at pH 7.0. The native TH behaved as the tetramer with an M_r of 240 000. In contrast, calpain-cleaved TH showed the monomeric M_r by gel permeation chromatography and increased K_i for catecholamine which inhibits the native TH in competition to the coenzyme, DL-6-methyl-5,6,7,8-tetrahydropterin ($6MPH_4$). These results imply that calpain cleavage would effectively release TH from the feedback inhibition by removal of the N-terminal region resulting in disruption of the quaternary structure.

Biosynthesis of catecholamines in catecholaminergic neurons and adrenal medullary cells is mainly regulated by the rate-limiting enzyme tyrosine hydroxylase [L-tyrosine,tetrahydropteridine:oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] (Nagatsu et al., 1964; Levitt et al., 1965), composed of four identical subunits with a molecular mass of approximately 60 kDa. TH[1] requires (6*R*)-L-*erythro*-tetrahydrobiopterin as a natural cofactor (Brenneman & Kaufman, 1964), and catecholamines inhibit TH in competition with the pterin cofactor (Nagatsu et al., 1964, 1972; Nagatsu, 1980). Recently, it has been shown that the phosphorylation sites (five Ser residues, Ser-8, Ser-19, Ser-31, Ser-40, and Ser-153, in the case of rat TH) exist in the N-terminal region (Campbell et al., 1986; Vulliet et al., 1989; Haycock, 1990). Among the five Ser residues, Ser-153 may not be a physiological phosphorylation site because of replacement of Ser by Ala in bovine TH and disappearance of the consensus sequence for cAMP-dependent phosphorylation in human TH (D'Mello et al., 1988). In addition, the limited tryptic proteolysis of the N-terminal region results in irreversible activation of TH (Abate et al., 1986; Bonnefoy et al., 1988). From these results, it was determined that TH consists of the inhibitory regulatory domain in the N-terminal region and the catalytic domain in the central through the C-terminal region. In any case, calpain alters the structures of proteins as well as their properties (Pontremoli et al., 1986). We had shown that both TH and calpain are present in the bovine adrenal medulla and that TH was activated after hydrolysis by calpain partially purified from the adrenal medulla (Togari et al., 1986). Therefore, TH may be modulated by limited proteolysis with calpain. To elucidate this problem, we examined changes in properties of TH cleaved by pure calpain to reveal the regulatory function of the N-terminal region in the TH molecule.

Experimental Procedures

Enzymes and Assays. TH was purified from bovine adrenal medulla as described by Kiuchi et al. (1987) by DEAE-Sephacel, Superose 6, and heparin–Sepharose chromatographies in the presence of protease inhibitors. TH activity was measured by an assay of DOPA (Nagatsu et al., 1979) using a high-performance liquid chromatograph with an electrochemical detector (Irika Amperometric Detector E-502). The standard assay mixture (200 μL) contained 40 nmol of L-tyrosine, 200 nmol of $6MPH_4$, 20 μmol of 2-mercaptoethanol, 40 μg of catalase, and enzyme in 0.1 M 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer (pH 7.0). The reaction was carried out at 37 °C for 10 min. Calpain was homogeneously purified from chicken skeletal muscle according to Ishiura et al. (1978). Protein concentrations were determined by a micro-assay using a Bio-Rad protein assay kit with bovine γ-globulin as a standard (Bradford, 1976).

Preparation of Monoclonal Antibody against TH. Spleen cells from BALB/c mice immunized with bovine TH were fused with P3-X63-Ag8-U1 myeloma cells using poly(ethylene glycol) 1500, and the hybridoma cells were prepared according to Köhler and Milstein (1975). The screening was carried out for the TH antigen by a combination of dot-immunobinding assay (Hawkes et al., 1982) and indirect immunoprecipitation using protein A–Sepharose. The clone 76/40/30 was selected from three positive clones since it gave the strongest immunostaining.

Electrophoresis and Western Blot Analysis. Samples were subjected to polyacrylamide gel electrophoresis in the presence

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[1] Abbreviations: TH, tyrosine hydroxylase; PDPK, proline-directed protein kinase; kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; $6MPH_4$, DL-6-methyl-5,6,7,8-tetrahydropterin; HPLC, high-performance liquid chromatography; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; pH, phenylalanine hydroxylase; TPH, tryptophan hydroxylase.

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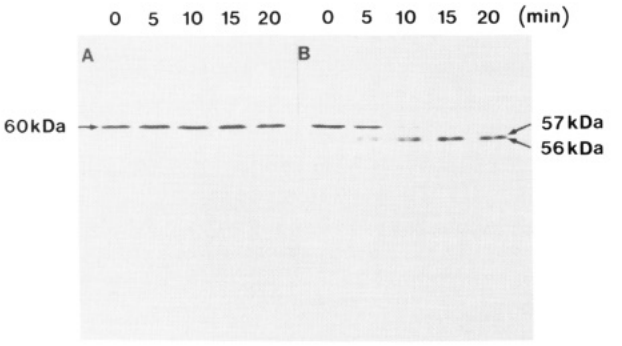

FIGURE 1: Time course of limited proteolysis of TH in the absence or presence of calpain by electrophoretic analysis. A total of 54 μg of TH was incubated in the reaction mixture (108 μL) in the absence (A) or presence (B) of calpain as described under Experimental Procedures. At each indicated time, a 10-μL aliquot was taken into a tube containing 0.5 μg of leupeptin, followed by the addition of 40 μL of 20 mM Tris-HCl buffer (pH 7.3) containing 8% sucrose, 1 mM dithiothreitol, and 1 mM EDTA. A 10-μL aliquot of each sample was subjected to SDS–PAGE.

of sodium dodecyl sulfate (SDS–PAGE) as described by Laemmli (1970) on 10% polyacrylamide gel. Gels were stained by the silver-staining technique (Oakley et al., 1980). Following electrophoresis, proteins were immediately transferred to a nitrocellulose sheet as described by Towbin et al. (1979). Intact and/or calpain-cleaved TH was detected on the sheet using monoclonal anti-bovine TH IgG and horseradish peroxidase conjugated goat anti-mouse IgG.

Proteolysis of TH with Calpain. The cleavage of TH was routinely performed at 25 °C in 20 mM Tris-HCl buffer (pH 7.0) containing 5 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM $CaCl_2$, and 0.1 mg/mL calpain. The proteolysis was terminated by addition of 0.5 μg of leupeptin/μg of calpain.

Gel Permeation Chromatography. A 50-μL sample of TH incubated in the reaction mixture for proteolysis with or without calpain was applied to a TSK G3000 SW glass column (8 × 300 mm) that had previously been equilibrated with 20 mM potassium phosphate buffer (pH 7.3) containing 8% sucrose, 1 mM DTT, 1 mM EDTA, 0.1 M KCl, and 0.1 μg/mL leupeptin. TH was eluted with the same buffer at a flow rate of 0.25 mL/min at 4 °C using an LKB HPLC system. The marker proteins used were glutamate dehydrogenase, lactate dehydrogenase, enolase, adenylate kinase, and cytochrome *c*. The elution positions of these proteins were determined by the absorbance at 280 nm, while that of TH was determined by its activity.

Amino Acid Sequence Analysis. The native TH (7.8 μg) and calpain-cleaved TH (25.8 μg) were applied to SDS–PAGE, respectively. Proteins were transferred to a Millipore poly(vinylidene difluoride) membrane according to Matsudaira (1987) and then directly subjected to amino acid sequence analysis with a Model 477A Applied Biosystems protein sequencer, equipped with an on-line PTH analyzer Model 120A using the manufacture's standard program.

Results and Discussion

When TH purified from bovine adrenal medulla was incubated in the presence of calpain purified from chicken muscle, the native TH (60 kDa) was rapidly degraded to 57- and 56-kDa molecules as shown by SDS–PAGE (Figure 1). After a 20-min incubation, the 60-kDa band was no longer detectable on SDS–PAGE (Figure 1). The anti-TH monoclonal antibody (76/40/30) recognized all of these 60-, 57-, and 56-kDa species (Figure 2). By the limited proteolysis with calpain, a slight but significant increase (1.3-fold) of TH activity at pH 7.0 was observed (Figure 3). It has been previously reported that

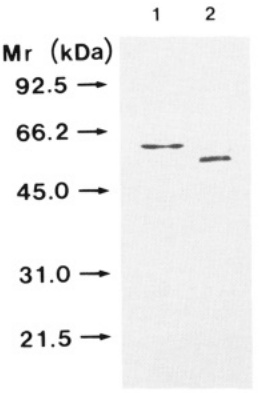

FIGURE 2: Western blotting of the native and calpain-cleaved TH. A 10-μL aliquot of each sample after incubation for 20 min in the absence or presence of calpain as described in the legend to Figure 1 was subjected to SDS–PAGE, followed by Western blotting as described under Experimental Procedures. Lane 1: without calpain. Lane 2: with calpain. Molecular weights of standard markers (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor) are indicated on the left.

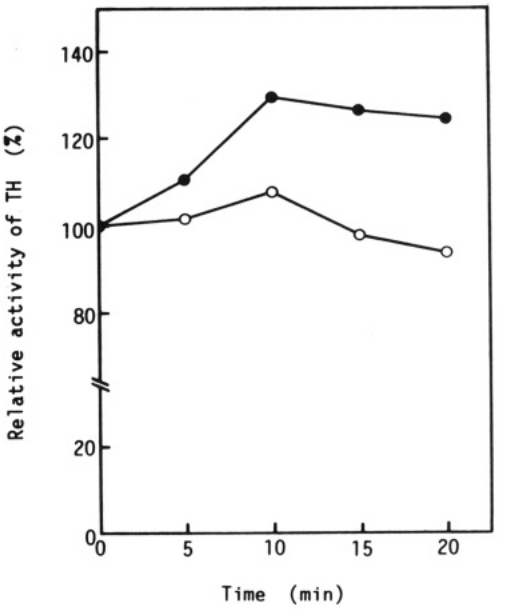

FIGURE 3: Time course of the enzymatic activity of TH incubated in the absence or presence of calpain. A 10-μL aliquot of each sample as described in the legend to Figure 1 was employed for the determination of the enzymatic activity of the native (○) and calpain-cleaved (●) TH at pH 7.0 as described under Experimental Procedures. TH activity at pH 7.0 at 0 min was 103 nmol/(min·mg of protein) and was taken as 100%.

TH activity is elevated 2-fold (Abate et al., 1986) or 3-fold (Bonnefoy et al., 1988) during the tryptic digestion. Indeed, limited proteolysis with calpain induced a similar shift in the pH optimum of tyrosine hydroxylation from 6.0 to 6.4 (Figure 4) as in the case of tryptic digestion (Vigny & Henry, 1981), but TH activity was not so remarkably elevated. This discrepancy may be explained by the fact that TH is cleaved in different manners with different proteases. Abate et al. (1986) have reported that the N-terminus of trypsin-digested TH from bovine adrenal medullary granules corresponds to residue 158 when compared to the amino acid sequence of rat TH deduced from the nucleotide sequence of its cDNA (Grima et al., 1985). This treatment produces a 34-kDa molecule which is smaller than that of calpain-cleaved TH. On the other hand, Bonnefoy et al. (1988) have reported that by a mild tryptic proteolysis for 10 min, rat TH (62 kDa) is digested to a 56-kDa molecule. This 56-kDa molecule lacks the N-terminal regulatory domain

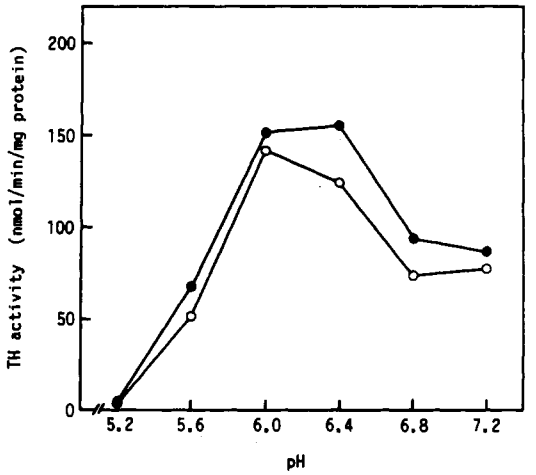

FIGURE 4: The pH profile of TH incubated in the absence or presence of calpain. A 10-μL aliquot of each sample as described in the legend to Figure 1 was employed for the determination of the enzymatic activity of the native (○) and calpain-cleaved (●) TH using MES buffer, pH 5.2–7.0, in the reaction mixture as described under Experimental Procedures.

Table I: Analysis of N-Terminal Sequences of Bovine TH before and after Cleavage by Calpain

	native TH		57-kDa molecule		56-kDa molecule	
cycle[a]	amino acid	pmol	amino acid	pmol	amino acid	pmol
1	Pro	13.0	Ala	2.28	Ser	4.74
2	Thr	5.0	Lys	0.44	Pro	5.30
3	Pro	15.0	Gln	2.39	Arg	2.36
4	Asn	11.4	Ala	1.61	Phe	3.34
5	Ala	13.2	Glu	1.34	Val	2.63

[a] For all species, sequencing was carried out for five cycles.

including Ser-8, Ser-19, Ser-31, and Ser-40 which can be phosphorylated with PDPK, kinase II, PDPK, and cAMP dependent protein kinase, respectively. In the present study, when TH was ^{32}P-labeled by kinase II and then cleaved by calpain, both the 56- and 57-kDa species showed no radioactivity as examined by the autoradiogram prepared after SDS–PAGE (data not shown). Since this result indicated that calpain cleaved TH in the proximity of the N-terminus, we determined the N-terminal sequences of the native (60-), 57-, and 56-kDa molecules (Table I). The N-terminus of the native TH corresponded to residue 2 (Pro), whereas those of the 57- and 56-kDa species corresponded to residue 23 (Ala) and residue 31 (Ser) of the deduced amino acid sequence (D'Mello et al., 1988), respectively, and both the 57- and 56-kDa species no longer had Ser-8 and Ser-19 phosphorylatable by kinases (Figure 5). As briefly described in our previous report by Togari et al. (1986), a drastic change took place in the quaternary structure of TH when TH was cleaved by calpain partially purified from bovine adrenal medulla. The M_r of TH cleaved by purified calpain was confirmed using high-performance gel permeation chromatography (Figure 6). Upon cleavage, TH became much smaller, perhaps comprising of monomers, whereas the native TH is a tetramer with an M_r of 240 000. This may suggest that the N-terminal region cleaved off by calpain may participate in constituting the quaternary structure of TH. From the concept that the N-terminal region constitutes the regulatory domain of TH containing four phosphorylation sites, we examined if calpain-cleaved TH exhibits a kinetical change against the coenzyme, pteridine. As shown in Figure 7, both the native and calpain-cleaved TH showed almost the same K_m value, 180 μM and 230 μM, respectively, toward $6MPH_4$. However, calpain cleavage caused a significant increase in K_i for dopamine and norepinephrine. K_i for dopamine increased from 4.4 μM to 72 μM, and that for norepinephrine increased from 2.3 μM to 17 μM. This indicates that limited proteolysis by calpain reduces the sensitivity of TH toward catecholamines. Petrack et al. (1968) reported that TH partially purified from the particulate fraction derived from adrenal medulla using trypsin for proteolysis was inhibited by catechol derivatives as well as the supernatant (native) enzyme. They examined the effect of catechol derivatives on TH activity by adding 0.5 mM norepinephrine or epinephrine as a final concentration to an incubation mixture. The concentration in their study was about 100-fold higher than the K_i for catecholamine, so the difference in inhibitory effect could not be observed. Roth et al. (1974) reported that the physiological concentration of dopamine was approximately 5 μM. Therefore, the increase in K_i by calpain cleavage would effectively release TH from the feedback inhibition as a short-term regulation.

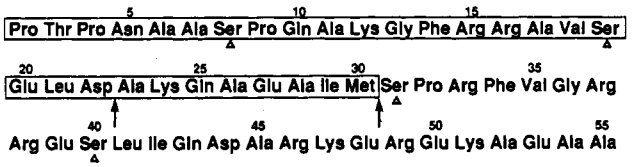

FIGURE 5: Cleavage sites of the native TH by calpain. Residues of the bovine TH molecule are numbered according to D'Mello et al. (1988). Arrows indicate the sites cleaved by calpain, and triangles indicate the putative phosphorylation sites. The region encoded by the first exon is illustrated by a box.

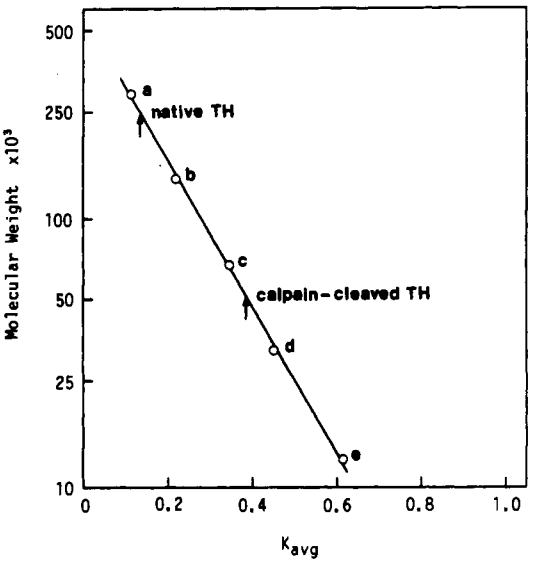

FIGURE 6: Molecular weight determination of both native and calpain-cleaved TH by high-performance gel permeation chromatography using a TSK G3000 SW glass column. The logarithm of the molecular weight was plotted versus distribution coefficient (K_{avg}). Molecular weight standards: a, glutamate dehydrogenase (290 kDa); b, lactate dehydrogenase (140 kDa); c, enolase (67 kDa); d, adenylate kinase (32 kDa); e, cytochrome *c* (12.4 kDa).

In the case of the 56-kDa species, the amino acid sequence encoded by the first exon of the bovine TH gene was completely missing, as judged by the structure of TH genes of rat (Brown et al., 1987) and human (O'Malley et al., 1987; Kobayashi et al., 1988). Brown et al. (1987) have compared the splice sites of rat TH gene with those of human phenylalanine hydroxylase (PH) gene to observe that the positions of 10 out of 12 intron/exon boundaries are identical. Darmon et al. (1988) have reported that a sequence homology is considerably high in the central through the C-terminal region (residues 187–324) among the rat aromatic amino acid hydroxylases, such as TH, PH, and tryptophan hydroxylase (TPH), i.e.,

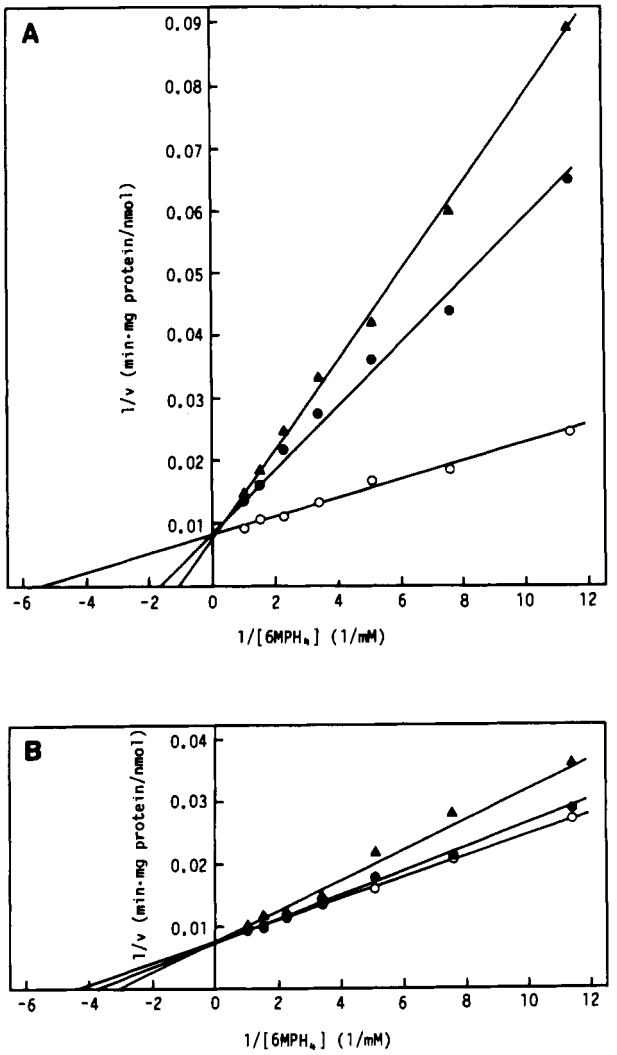

FIGURE 7: Kinetic comparison of the enzymatic activity of the native and calpain-cleaved TH. A 10-μL aliquot of each sample as described in the legend to Figure 2 was employed for the determination of TH activity. The enzymatic activity of the native (A) and calpain-cleaved (B) TH was measured in the absence (○) or presence (●, ▲) of 10 μM dopamine and norepinephrine, respectively. The assay mixture contained 0.2 mM L-tyrosine and $6MPH_4$ from 87.8 μM to 1 mM.

76.1% between TH and PH, 73.3% between TPH and PH, and 71.8% between TPH and TH, whereas the homology is weak in the N-terminal region among the three enzymes. Therefore, the active site domains of these hydroxylases must have been evolved from a common ancestor gene, and each N-terminal domain has perhaps been attached to the homologous domain as a regulatory domain through the evolution. The N-terminal region of the TH molecule can be regarded as a regulatory domain exerting a negative regulation in the enzymatic activity, and the region encoded by the first exon of TH gene should be a portion responsible for the feedback inhibition by catecholamine. At present, little is known about functional significance of the regions of the TH molecule encoded by the second exon, the third exon, and so on. However, it would be interesting to know how many exons are needed for the appearance of the enzymatic activity. With the advent of new techniques in molecular biology, such as deletion of some exon or site-directed mutagenesis, the functions of the N-terminal region of these hydroxylases could be further clarified.

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Registry No. TH, 9036-22-0; $6MPH_4$, 58936-36-0; calpain, 78990-62-2; dopamine, 51-61-6; norepinephrine, 51-41-2.

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