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Transient Activation of Dihydropteridine Reductase by Ca^{2+}-activated Proteolysis

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ABSTRACT—A transient activation of dihydropteridine reductase (DHPR), which is the regenerating enzyme of tetrahydrobiopterin in the system of aromatic amino acid hydroxylases, was studied during the incubation of DHPR with Ca^{2+}-activated protease, m-calpain. The DHPR subunit (29 k) was cleaved by m-calpain just before the 35th (Ser) and 48th (Val) residue from the N-terminus, generating two new fragments of 21 k and 19 k. By determining kinetic parameters, we found that 21 k and 19 k were more active than the native enzyme and that the activation of them was more remarkable and transient against the natural substrate of quinonoid dihydrobiopterin than against a synthesized substrate. Phosphorylation of DHPR by Ca^{2+}/calmodulin-dependent protein kinase II controlled the sensitivity of the enzyme to the Ca^{2+}-activated protease.

INTRODUCTION

Tetrahydrobiopterin (BH4) functions as a H-donor cofactor of aromatic amino acid hydroxylases (Nagatsu *et al.*,1972; Kaufman and Fisher, 1974), rate-limiting enzymes for producing monoamine neurotransmitters in the sympathetic nervous system, and becomes converted to quinonoid dihydrobioptrin (q-BH$_2$) through 4α-carbinolamine in the process. The regeneration of BH$_4$ from q-BH$_2$ by dihydropteridine reductase (DHPR; NADH: quinonoid dihydropteridine oxidoreductase [EC 1. 6. 99. 7]) with NADH allows this cofactor to function catalytically. Besides the biosynthesis of BH$_4$ from GTP by three enzymes (Katoh and Akino, 1986), GTP cyclohydrolase I (Hatakeyama *et al.*, 1989), 6-pyruvoyl-tetrahydropterin synthase (Inoue *et al.*, 1991), and sepiapterin reductase (SPR) (Sueoka and Katoh, 1982), the BH$_4$-recycling by DHPR is important to control the concentration of the cofactor in the cell. DHPR reduces with NADH various quinonoid dihydropterins derived from BH$_4$ and other tetrahydropterins such as 6-methyl tetrahydropterin (6M-PH$_4$). DHPR has been purified to homogeneity from many sources (Craine *et al.*, 1972; Hasegawa, 1977; Korri *et al.*, 1977; Webber *et al.*, 1978; Firgaira *et al.*, 1981). The cDNAs of human (Dahl *et al.*, 1987), mouse (Yang *et al.*, 1996), and rat (Shahbaz *et al.*, 1987) DHPR have been cloned, and their deduced amino acid sequences are almost identical.

The calcium ion plays a role as a second messenger with a regulatory involvement in many aspects of cellular signaling and regulates the activation of various protein kinases (Greengard, 1978) and proteases such as calpain. Recently, both DHPR and SPR, which are BH$_4$-recycling enzyme and BH$_4$-generating enzyme, respectively, were found to be phosphorylated by Ca^{2+}-activated protein kinases (Katoh *et al.*, 1994). Calpain is a Ca^{2+}-activated protease widely distributed in various organs such as skeletal muscle (Huston *et al.*, 1968; Dayton *et al.*, 1976), platelets (Phillips and Jakabova, 1997), axoplasm (Schlaepfer, 1974; Pant *et al.*, 1979), liver (Nishiura *et al.*, 1978), and heart (Waxman and Krebs, 1978; Drummond and Duncan, 1966). Calpain is inhibited by an endogenous inhibitor, calpastatin (Nishiura *et al.*, 1978). It is wellknown that calpain has a strict substrate specificity, and modulates functions of various physiologically active proteins by limited proteolysis (Suzuki, 1993).

We found, in this study, that DHPR is transiently activated by limited proteolysis by m-calpain.

MATERIALS AND METHODS

Chemicals

DHPR (sheep liver), Ca^{2+}-activated protease (m-calpain), calpastatin, ferri-cytochrome c, 2,6-dichlorophenolindophenol (DCPIP), ATP, calmodulin, ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetra acetic acid (EGTA) and protein markers were obtained from Sigma (U.S.A.). Nicotinamide adenine dinucleotide (reduced form; NADH) was obtained from Wako Pure Chem. (Japan), and polyvinylidene difluoride (PVDF) membrane (Immobilon-P^{SQ}), from Millipore (U.S.A.). Tetrahydrobiopterin (BH$_4$) and 6-methyl-5, 6, 7, 8-tetrahydropterin (6M-PH$_4$) came from Dr. Schricks Lab. (Switzerland). Sepiapterin reductase (SPR) and Ca^{2+}/calmodulin-dependent protein kinase II were prepared from hemolysate (Sueoka and Katoh, 1982) and cerebral cortex (Yamauchi and Fujisawa, 1983), respectively, obtained from rats.

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DHPR assay

The commercially obtained DHPR was further purified by HPLC using a Shodex DEAE-825 column (5×100 mm). DHPR was eluted by a linear gradient of 0–0.15 M KCl in 20 mM phosphate buffer, pH 7.5, at a flow rate of 0.5 ml/min. As the substrate of DHPR, 6-methyl quinonoid dihydropterin (q-MH_2) was generally prepared from 6M-PH4 by non-enzymatic oxidation with ferri-cytochrome c just before use. The amount of substrate reduced by DHPR in the presence of NADH was determined spectrophotometically as the amount of ferro-cytochrome c (Katoh *et al.*, 1970). The reaction mixture contained 50 mM Tris-HCl, pH 7.5, 0.05 mM ferri-cytochrome c, 0.05 mM NADH, 1 µM 6M-PH_4, and 1 µg DHPR in a final volume of 2 ml. The mixture without DHPR was incubated for 1 min after 6M-PH_4 had been added to form q-MH_2, and then the reaction was started by the addition of DHPR and monitored for 1 min at 550 nm (Hitachi Spectrophotometer U-3210). Assays were performed at 25°C. One unit (U) was defined as 1 µmole of cytochrome c reduced per min (Hasegawa, 1977). Concentration of tetrahydropterin was determined by spectrophotometric titration with DCPIP (Kaufman and Levenberg, 1959).

Proteolysis of DHPR by Ca^{2+}-activated protease (m-calpain)

DHPR was incubated with m-calpain (0.05–1 U) in the reaction mixture containing 50 mM Tris-HCl (pH 7.5), 5 mM $CaCl_2$, and 5 mM 2-mercaptoethanol for 5–180 min at 25°C. After the incubation, SDS for a 0.1% final concentration was added to the reaction mixture, which was then boiled for 3 min. The resultant solution was applied to SDS-PAGE (12.5% gel) performed according to Laemmli (Laemmli, 1970). Proteins on the gel were stained with Coomassie brilliant blue G-250.

Analysis of N-terminal amino acid sequence

After electrophoresis, separated proteins on the gel were transferred to a PVDF membrane at 400 mA for 3 hr in 100 mM sodium tetraborate. The transferred protein fragments on the PVDF membrane stained with Coomassie brilliant blue G-250 were cut out and directly applied to a protein sequencer. The N-terminal amino acid sequences of the fragments were determined by the Edman method (Edman and Begg, 1967) with a gas-phase protein sequencer (Applied Biosystems model 477A) with the chemicals and program supplied by the manufacturer.

Phosphorylation of DHPR

DHPR (5 µg) was added to a reaction mixture (final 100 µl) containing 50 mM Tris-HCl buffer (pH 7.5), 0.5 µg Ca^{2+}/calmodulin-dependent protein kinase II, 0.5 µM calmodulin, 100 µM ATP, 2 mM magnesium acetate, and 0.15 mM $CaCl_2$, and incubated at 25°C for 10 min, under which conditions DHPR is maximally phosphorylated (Katoh *et al.*, 1994).

Protein measurement

Protein concentration was determined by measuring its absorbance at 280 nm with bovine serum albumin as a standard (Hitachi Spectrophotometer U-3210).

RESULTS

Activation of DHPR by m-calpain

Effects of m-calpain and calcium ion on the activity of DHPR were observed *in vitro*. As shown in Fig. 1a, a transient activation of DHPR was observed during the initial period of the incubation of DHPR with m-calpain in the presence of calcium ions. DHPR activity increased to about 150% after the incubation for 5 through 30 min, and then it decreased gradually; however, about 120% activity was maintained until 180 min. This activation of DHPR was not observed when
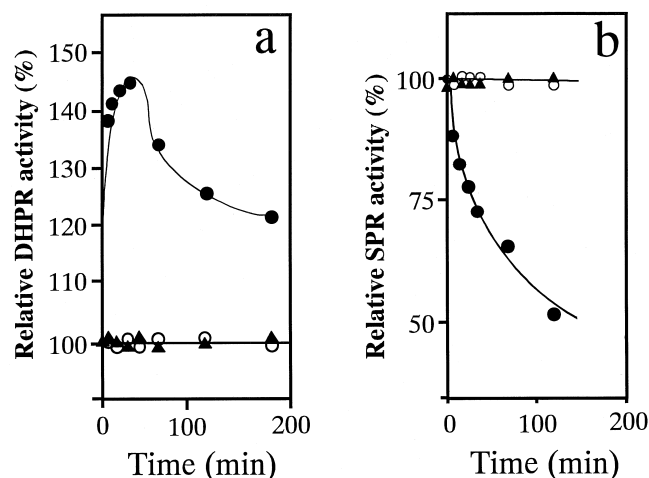
Fig. 1. Time-dependent activation of DHPR by m-calpain. (a) DHPR (10 µg) was incubated with m-calpain (1 U) in the presence ( ) or absence ( ) of calpastatin (1 U) in the reaction mixture containing 5 mM $CaCl_2$, 5 mM 2-mercaptethanol, and 50 mM Tris-HCl buffer (pH 7.5) in a final volume of 100 µl at 25°C. At various times, an appropriate volume containing 1 µg DHPR was removed from the reaction mixture and measured for activity as described in the text. DHPR was incubated without m-calpain and calpastatin ( ) as a control. (**b**) SPR was incubated in the presence or absence of m-calpain and calpastatin as described in (**a**), and assayed for activity by a published method (Katoh *et al.*, 1994).
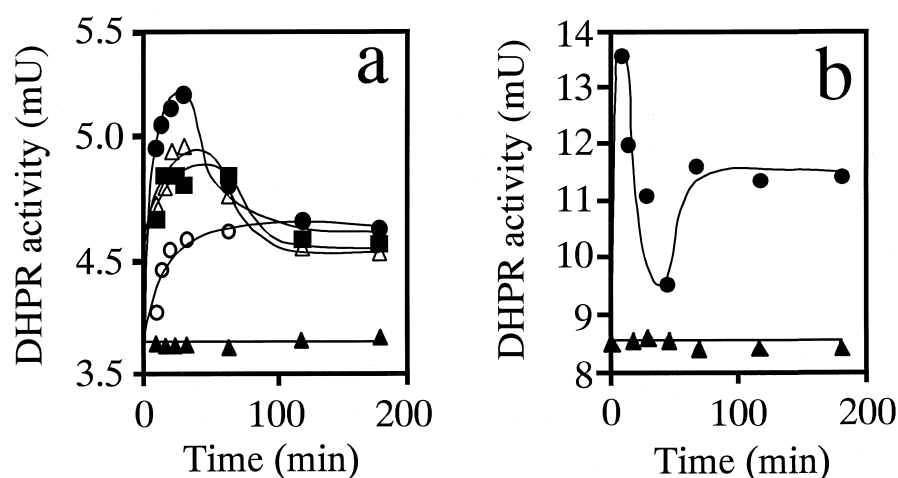
Table 1. Effect of calcium ion on the activation of DHPR by m-calpain

Addition	DHPR activity (mU)	[%]
none	3.67	100
m-calpain	3.46	94
m-calpain+Ca^{2+}	5.21	142
m-calpain+Ca^{2+}+EGTA	3.39	92
m-calpain+EGTA	3.39	92
m-calpain+Ca^{2+} (5 min) +EGTA	3.78	103

DHPR (6 µg) and m-calpain (1 U) were preincubated in the presence or absence of calcium ion and/or EGTA in the reaction mixture at 25°C for 5 min as described in Fig. 1a. In the last experiment in the table, the reaction mixture was treated with EGTA after preincubation of DHPR and m-calpain with calcium ions and further incubated for 5 min. DHPR equivalent to 1 µg of the reaction mixture was assayed for activity by the method described in the text.

DHPR was incubated with m-calpain in the presence of calpastatin, an endogenous specific inhibitor of m-calpain (Nishiura *et al.*, 1978). However, activation of DHPR by m-calpain was not observed when calcium ions were omitted before or after the incubation (Table 1). These results indicate that DHPR was activated by m-calpain that was stimulated by calcium ion and that calcium ions were also necessary for activation of DHPR after the proteolytic function of m-calpain. On the other hand, although SPR was also a substrate of m-calpain, the activity of SPR was decreased by the incubation with m-calpain (Fig. 1b).

Limited proteolysis of DHPR by m-calpain

DHPR is a homodimeric enzyme (Webber *et al.*, 1978), and the molecular weight of the subunit was estimated by SDS-PAGE as 29 k in this experiment. Proteolytic effect of m-calpain on DHPR molecule was analyzed by SDS-PAGE. When DHPR was incubated with m-calpain in the presence of calcium ion, two new fragments A (21 k) and B (19 k) were initially produced from the DHPR subunit (29 k); but only fragment B was observed after 120 min (Fig. 2). The latter finally disappeared,

too, by further incubation. These findings indicate that the appearance of fragments A and B were dependent on limited proteolysis with m-calpain and that fragment A seemed to decompose faster than fragment B.

Hydrolyzed sites of DHPR by m-calpain

After the incubation of DHPR with m-calpain for 5 min as shown in Fig. 2, fragments A and B were isolated by SDS-PAGE for analysis with a protein sequencer. The amino acid

Fig. 2. Time-dependent proteolysis of DHPR by m-calpain. DHPR was incubated with m-calpain (1 U) at 25°C for 5 or 120 min as described in Fig. 1, and an appropriate volume containing 10 μg DHPR was applied to SDS-PAGE as described in the text. The leftmost lane indicates protein markers. Lanes 1, 2, and 3 indicate reaction mixtures incubated for 0, 5, and 120 min, respectively. Proteins on the gel were stained with Coomassie brilliant blue G-250.

Fig. 3. Determination of cleavage sites of DHPR by m-calpain. (**a**) N-terminal amino acid sequences of fragment A and B determined by Edmen method (Edman and Begg, 1967) as described in the text with reference to the sequences of DHPR from various origins (Dahl *et al.*, 1987; Yang *et al.*, 1996; Shahbaz *et al.*, 1987). Asterisks indicate N-terminal sites of fragment A (*) and fragment B (**), respectively. (**b**) The position of cleavage sites in DHPR subunit. The X-ray data for rat DHPR were cited from the Protein Data Bank (PDB code 1DHR). Numbers 146 and 150 indicate the catalytic motif of DHPR (Tyr^{146}-X-X-X-Lys^{150}) for the pteridine substrate. The nicotinamide moiety of NADH is also located around the motif according to the data.

Fig. 4. Activation patterns of DHPR by various amounts of m-calpain by adding 6M-PH$_4$ or BH$_4$ as the source of quinonoid dihydropterin substrate. DHPR (10 μg) was incubated with various amounts of m-calpain for various times at 25°C in the system (final 100 μl) of m-calpain as described in the text (1 U [ ], 0.5 U [ ], 0.25 U [ ], 0.05 U [ ], none [ ]). After various times of incubation, the activity of DHPR (1 μg equivalent) was measured for 1 min in the system described in the text against q-MH$_2$ (**a**) and q-BH$_2$ (**b**).

sequence of sheep DHPR was previously analyzed except for about 100 amino acids in its N-terminal region (Lockyer *et al.*, 1987). The N-terminal sequences of fragments A and B analyzed in this study were determined as Ser-Ile-Asp-Val-Gln-Glu-Asn-Glu and Val-Val-Val-Lys-Met-Thr-Asp-Ala-Phe-Leu, respectively (Fig. 3a). These sequences correspond to the N-terminal regions around about 30 - 60th of DHPR subunits of rat (Shahbaz *et al.*, 1987), mouse (Yang *et al.*, 1996), and human (Dahl *et al.*, 1987) enzymes, as shown in Fig. 3a. These results indicate that the DHPR subunit was cleavaged by m-calpain between Ala-34 and Ser-35 and between Ser-47 and Val-48 to form fragments A and B, respectively (Fig. 3b).

Activities of fragments A and B

Fig. 4a shows time courses of the activities of DHPR against q-MH$_2$ during the incubation with various amounts of m-calpain. When DHPR was incubated for 5 min with 1 U m-calpain, the activity increased to about 150% of that of the native DHPR. The activity gradually decreased to about the 120% level, and this level remained until 180 min after the start of incubation. When a much smaller amount of m-calpain (0.05 U) was added, DHPR activity increased to about the 120% level, and it remained there until 180 min. These data (Fig. 4a) and the results by SDS-PAGE (Fig. 2) show that fragment A was more easily decomposed than fragment B and had higher activity than native DHPR or fragment B and that fragment B had higher activity than native DHPR. If the natural substrate (q-BH$_2$) of DHPR was used by the addition of BH$_4$, in the assay system of DHPR, a more rapid increase and decrease in DHPR activity was observed than with the chemically synthesized substrate (q-MH$_2$) during the initial time of incubation of DHPR and m-calpain, as shown in Fig. 4b.

Modulation of catalytic properties of DHPR by limited proteolysis

Fig. 4b also shows that native DHPR has higher activity toward q-BH$_2$ than toward q-MH$_2$. To know the differences in the catalytic properties between the native and modified DHPR, we analyzed the activities in the reaction mixture with m-calpain after the incubations for 5 min as the mixture of fragments A and B, and native DHPR, and for 120 min as fragment B. Fragments A and B and native enzyme could not be separated from each other by HPLC in suficient amount for the experiment because of further proteolysis during application of the mixture onto the column and because of similarity in molecular sizes of these fragments. As shown in Table 2, the sample incubated for 5 min had smaller Kms for both substrates than that containing fragment B (120 min-incubation), and fragment B had smaller ones than the native DHPR (0 min-incubation). All fragments show lower values in Km and larger values in Vmax/Km when the natural substrate q-BH$_2$ was used than when the chemically synthesized q-MH$_2$ was the substrate. Especially, the Vmax/Km value for q-BH$_2$ of the 5-min sample was about 2 fold that of the native DHPR (Table 2). These results indicate that fragment A has higher affinity and utilization for the natural substrate q-BH$_2$ than fragment B or native DHPR.

Table 2. Effect of m-calpain treatment on the kinetics of DHPR

Incubation time	Km (μM)		Vrel/Km	
	q-MH$_2$	q-BH$_2$	q-MH$_2$	q-BH$_2$
0	3.2	2.5	0.15 [100]	0.19 [100]
5 min	2.7	1.1	0.18 [120]	0.35 [184]
120 min	3.0	1.7	0.16 [107]	0.25 [132]

DHPR was preincubated with m-calpain and calcium ion for 5 or 120 min and determined in kinetic parameters as described in Fig. 5. DHPR equivalent to 1 μg of the preincubation mixture was assayed for activity against various amounts of q-MH$_2$ and q-BH$_2$ as described in Fig. 5. Vrel; relative Vmax.
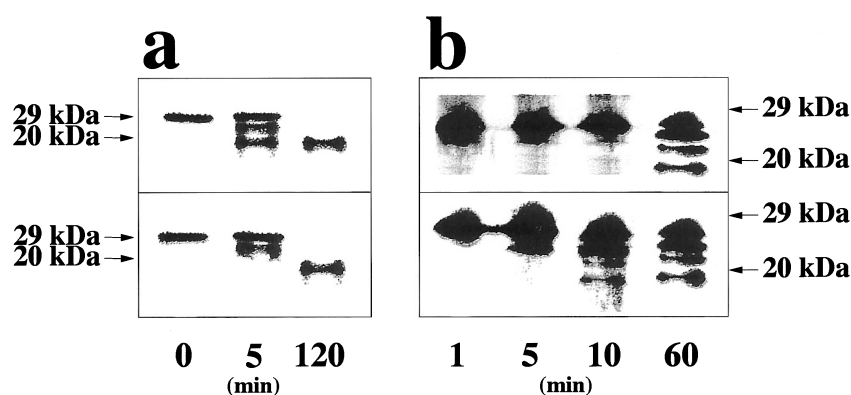
Fig. 5. Effect of phosphorylation of DHPR on the sensitivity to m-calpain. SDS-PAGE of phosphorylated and non-phosphorylated enzymes after being incubated with m-calpain. DHPR or SPR was phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II as described in the text. The reaction mixture was further incubated with 1 U of m-calpain for various times, and an appropriate volume containing 10 µg DHPR and 30 µg SPR was applied to SDS-PAGE. (**a**) DHPR phosphorylated (lower) and non-phosphorylated (upper). (**b**) SPR phosphorylated (lower) and non-phosphorylated (upper).

Sensitivity of DHPR to m-calpain

DHPR and SPR can be phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II, as described in our previous report, although these enzymes are hardly affected in terms of their catalytic properties (Katoh *et al.*, 1994). During the incubation of DHPR with m-calpain, besides the native DHPR, fragments A and B were observed after 5 min; whereas only fragment A was seen at that time when DHPR was incubated after having been phosphorylated with Ca^{2+}/calmodulin-dependent protein kinase II according to the method described previously (Katoh *et al.*, 1994) (Fig. 5a [lower panel]). This indicates that DHPR became resistant to Ca^{2+}-activated protease by Ca^{2+}-dependent phosphorylation. SPR was also found to be cleaved by m-calpain (Fic. 5b [upper panel]), although its activity decreased with incubation time, as shown in Fig. 1b. Three visible fragments of 26 k, 24 k, and 19 k were formed when the native SPR (28 k) was incubated with m-calpain for 60 min (Fig. 5b [upper]). If SPR was incubated with m-calpain after the former had been phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II, these three fragments were observed even after 10 min (Fig. 5b [lower]). Accordingly, in the case of SPR, sensitivity to m-calpain was accelerated by Ca^{2+}-dependent phosphorylation. Sensitivity of DHPR or SPR to V8 protease, which does not require calcium ions for its activity, however, was not affected by phosphorylation with Ca^{2+}/calmodulin-dependent protein kinase II (data not shown).

DISCUSSION

In this study, we found a transient activation of DHPR by m-calpain, a widely distributed Ca^{2+}-activated protease, *in vitro*. The activation of DHPR was observed during the initial period of the incubation of DHPR with m-calpain in the presence of calcium ions (Fig. 1a). By SDS-PAGE of the reaction mixture, two new fragments of 21 k and 19 k appeared from the native DHPR subunit of 29 k during the incubation (Fig. 2). These fragments on the gel were isolated, and their N-terminal amino acid sequences were analyzed by the Edman method. The result indicated that the DHPR subunit was cleaved between Ala-34 and Ser-35 and between Ser-47 and Val-48 by m-calpain, and converted to 21 k and 19 k fragments, respectively (Fig. 3a). The positions of the hydrolysis by m-calpain in DHPR subunit were indicated on the three-dimensional structure obtained from X-ray data cited from the Data Bank (Su *et al.*, 1993) (Fig. 3b)

The two fragments were more active than the native DHPR subunit. Fragment A was the most active type, since the activity in the 5-min incubation sample, in which fragments A and B and native DHPR were present (Fig. 2), was higher than that in the sample taken at 0 time (native DHPR) or 120 min (fragment B) (Fig. 2, Table 2). Vrel/Km values of these fragments were larger than those of native DHPR (Table 2). It is physiologically significant that the rate of activation of these fragments was more remarkable when the natural cofactor, BH_4 was used as the source of the substrate than when the synthesized cofactor, $6M\text{-}PH_4$ was used (Table 2). Like SPR (Katoh and Sueoka, 1984; Sueoka and Katoh, 1985), DHPR was demonstrated to be a member of the short-chain dehydrogenase/reductase superfamily, based on its NAD(P)H requirement and primary structure (Jornvall *et al.*, 1995). The enzymes of this family generally have a coenzyme-binding domain in their N-terminal region and a unique segment in their C-terminal region, for individual function, containing conserved residues of the essential motif Tyr-X-X-X-Lys. As well as SPR (Tyr[171]-X-X-X-Lys[175]), DHPR contains a specific Tyr[146]-X-X-X-Lys[150] motif in its sequence, and the pteridine substrate and nicotinamide ribose moiety of NADH were located near this sequence by crystallographic analysis (Su *et al.*, 1993). These data and the results in this study indicate that cleavages of DHPR in the N-terminal region (34–35 and 47–48 in the sequence) by m-calpain in the presence of calcium ions modified the conformational structure around the active site of the enzyme and may spread out the domain around the pteridine site to increase the affinity strictly for the natural pteridine substrate (Fig 3b and Fig 4b). Small fragments of the

N-terminus cut off by m-calpain, however, might still have been connected to fragment A or B in the presence of calcium ions since the activation was not observed when EGTA was added after the proteolytic reaction (Table 1).

m-Calpain has a strict substrate specificity, and bring about functional modulation of various proteins (Suzuki, 1993). DHPR and SPR were found to be good substrates for m-calpain in this study, and limitedly proteolyzed by the protease. DHPR showed a transient increase in its activity due to the proteolysis, whereas, SPR, in contrast, displayed a decreased activity, as was shown in Fig. 1a, b. The SPR subunit (28 k) was cleaved to at least three fragments of 26 k, 24 k, and 19 k during the initial incubation with m-calpain, as indicated in Fig. 5b; however, only one site of Val187 was detected by analysis of the N-terminal amino acid (data not shown). This result suggests that SPR was cleaved by m-calpain at its C-terminal region, which is located near the catalytic site of Tyr[171]-X-X-X-Lys[175], the motif contributing to the enzyme activity (Fujimoto *et al.*, 1999), and then lost its activity. The sites of hydrolysis of DHPR and SPR by m-calpain found in this study however, were located in the sequences indicating negative scores for PEST sequences for the calpain reaction, as obtained by PESTfind Analysis (Rogers *et al.*, 1986; Rechsteiner and Rogers, 1996). The finding shown in Fig. 5a, b, in which the cleavage of DHPR by Ca^{2+}-activated protease is controlled by phosphorylation by Ca^{2+}-dependent protein kinase, seems to be significant *in vivo*. Tyrosine hydroxylase, a BH_4-requiring enzyme for formation of catecholamines, is also limitedly proteolyzed and increased in its activity by m-calpain (Kiuchi *et al.*, 1991). As well as SPR and DHPR (Katoh *et al.*, 1994), tyrosine hydroxylase (Campbell *et al.*, 1986) and nitric oxide synthase (Bredt *et al.*, 1992) are also phosphorylated by Ca^{2+}/calmodulin-dependent protein kinase II and/or protei kinase C. It is physiologically understandable that enzymes involved in the biosynthesis and recycling of BH_4, and those requiring it might all be controlled by calcium ions through phosphorylation and proteolysis to regulate the amounts of neurotransmitters such as catecholamines, indoleamines, and nitric oxide in the cell.

ACKNOWLEDGMENTS

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