Identification of the domains of neuronal nitric oxide synthase by limited proteolysis

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Nitric oxide synthase (EC 1.14.13.39) binds arginine and NADPH as substrates, and FAD, FMN, tetrahydrobiopterin, haem and calmodulin as cofactors. The protein consists of a central calmodulin-binding sequence flanked on the N-terminal side by a haem-binding region, analogous to cytochrome *P*-450, and on the C-terminal side by a region homologous with NADPH:cytochrome *P*-450 reductase. The structure of recombinant rat brain nitric oxide synthase was analysed by limited proteolyis. The products were identified by using antibodies to defined sequences, and by N-terminal sequencing. Low concentrations of trypsin produced three fragments, similar to those in a previous report [Sheta, McMillan and Masters (1994) J. Biol. Chem. **269**, 15147–15153]: that of M_r approx. 135000 (N-terminus Gly-221) resulted from loss of the N-terminal extension (residues 1–220) unique to neuronal nitric oxide synthase. The fragments of M_r 90000 (haem region) and 80000 (reductase region, N-terminus Ala-728) were produced by cleavage within the calmodulin-binding region. With more extensive trypsin treatment, these species were shown to be transient, and three smaller, highly stable fragments of M_r 14000 (N-terminus Leu-744 within the calmodulin region), 60000 (N-terminus Gly-221) and 63000 (N-terminus Lys-856 within the FMN domain) were formed. The species of M_r approx. 60000 represents a domain retaining haem and nitroarginine binding. The two species of M_r 63000 and 14000 remain associated as a complex. This complex retains cytochrome *c* reductase activity, and thus is the complete reductase region, yet cleaved at Lys-856. This cleavage occurs within a sequence insertion relative to the FMN domain present in inducible nitric oxide synthase. Prolonged proteolysis treatment led to the production of a protein of M_r approx. 53000 (N-terminus Ala-953), corresponding to a cleavage between the FMN and FAD domains. The major products after chymotryptic digestion were similar to those with trypsin, although the pathway of intermediates differed. The haem domain was smaller, starting at residue 275, yet still retained the arginine binding site. These data have allowed us to identify stable domains representing both the arginine/haem-binding and the reductase regions.

INTRODUCTION

Nitric oxide synthase [L-arginine,NADPH:oxygen oxidoreductase (nitric-oxide-forming); EC 1.14.13.39; NOS] catalyses the conversion of arginine, oxygen and NADPH into citrulline, nitric oxide and $NADP^+$ [1–4]. There are three main isoforms of the enzyme: neuronal (nNOS), endothelial (eNOS) and inducible (iNOS), all of which contain bound haem, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin), FMN and FAD, and interact with calmodulin. Their amino acid sequences show extensive regions of similarity, reflecting their common catalytic function and cofactor requirements [1,3]. The enzymes are composed of three main regions: a C-terminal half, which contains the FMN, FAD and NADPH binding sites, a central calmodulin-binding site and an N-terminal region, which contains the haem moiety, and probably the arginine and tetrahydrobiopterin sites [5–9] (Figure 1). The amino acid sequence of the C-terminal half has significant identity with cytochrome *P*-450 reductase whereas that of the N-terminal region does not have a high similarity with other proteins, but appears to be analogous to a cytochrome *P*-450. The binding sites for arginine, tetrahydrobiopterin and haem have not yet been closely defined, although it is likely that the haem is bound to Cys-415 [10,11]. The calmodulin-binding site has been identified as a stretch of about 23 amino acids [6,7]. Unique to nNOS is an N-terminal extension of about 200 amino acids containing the Discs-large Homologous Region, which might be important in its intracellular localization [12]. Rat nNOS is a protein of M_r 160558 (p160) containing 1429 residues (Figure 1).

In terms of overall enzyme reaction and structure, NOS most closely resembles the fatty acid ω-hydroxylase (cytochrome *P*-450 BM3) enzyme from *Bacillus megaterium*, which catalyses the NADPH- and oxygen-dependent hydroxylation of fatty acids in a system using FAD, FMN and haem as cofactors [13–16]. This reaction is formally analogous to the first partial reaction catalysed by NOS, i.e. hydroxylation of arginine to form hydroxy-

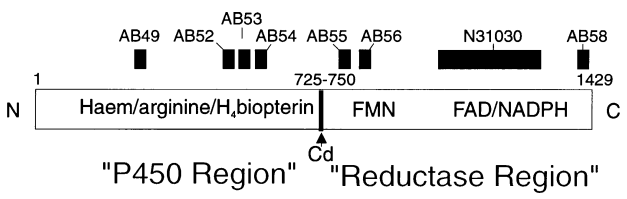

Figure 1 The primary structure of rat brain nNOS

The primary structure of rat brain nNOS and the regions within which are binding sites for its substrates and cofactors are depicted. The locations of the peptide sequences that were used to raise the antibodies employed in these studies are indicated. Abbreviation: H_4biopterin, tetrahydrobiopterin.

Abbreviations used: Cd, calmodulin; tetrahydrobiopterin, (6*R*)-5,6,7,8-tetrahydro-L-biopterin; nitroarginine, N^{ω}-nitro-L-arginine; NOS, nitric oxide synthase; nNOS, eNOS, iNOS, neuronal, endothelial and inducible forms of NOS.

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arginine. The ω-hydroxylase differs from NOS in that it is not regulated by calmodulin and has no requirement for tetrahydrobiopterin. The domain structure of BM3 ω-hydroxylase has been studied by limited proteolysis [15,17], and this has allowed recombinant domains to be expressed, characterized and their X-ray structure determined [18–21].

Recently, reports that nNOS and iNOS have bidomain structures were published, based in part on limited proteolysis [8,9]. In those studies, NOS was partially digested with trypsin to give a predominant cleavage in the calmodulin-binding site, but proteolysis was not continued to completion. In this report we describe a more extensive proteolysis in conjunction with a functional assay for the arginine site, and a range of site-selective antibodies, to define more closely the functional domain structure of rat nNOS. This has allowed us to identify stable domains representing both the arginine/haem-binding and the reductase regions.

EXPERIMENTAL

Antibodies

N31030 antibody to nNOS (affinity purified, rabbit polyclonal, raised against residues 1095–1290 of human nNOS, corresponding to residues 1091–1286 of rat nNOS with five amino acid substitutions, which span parts of the FAD and NADPH binding motifs) was made by Transduction Labs (Cat. No. N31030) and supplied by Affiniti research products, Nottingham, U.K. The following rabbit anti-sera, raised as described in [22], were used: antibodies to rat nNOS peptides 49 (residues 251–270), 52 (481–500), 53 (520–540), 54 (545–565), 55 (781–801), 56 (845–864) and 58 (1409–1429). These are designated AB49, AB52, AB53, AB54, AB55, AB56 and AB58, respectively. A mixture in equal volumes of sera AB 52, AB53 and AB54 is referred to as AB52-54. For Western blotting, antisera were used at a dilution of 1 in 6000.

[^{3}H]Nitroarginine binding

N^{ω}-Nitro-L-[2,3,4,5-^{3}H]arginine (Amersham) was mixed with unlabelled nitroarginine (Sigma) to obtain a specific activity of 7200 Ci/mol. NOS (1–10 μg) was incubated with 0.7 μM [^{3}H]nitroarginine in 50 mM Tris/HCl, pH 7.5, containing 10 μM tetrahydrobiopterin and 1 mM dithiothreitol in a total volume of 50 μl for 30 min at 22 °C. The sample was diluted to 1 ml with ice-cold 50 mM Tris/HCl, pH 7.5, and immediately filtered through a nitrocellulose filter (BA85, Schleicher & Schuell). The filter was washed with 8 ml of ice-cold buffer, dissolved in Filtercount scintillant (Packard) and counted in a liquid-scintillation counter. Blanks, in which no protein was present, were subtracted to give the amount of specific binding. Blanks were unaffected by the inclusion of 0.2 mg/ml BSA. Typically, blanks were about 300 d.p.m. and assays containing 1 μg of nNOS gave 18000 d.p.m.

Electrophoresis and Western blotting

SDS/PAGE was performed on gels containing either 19.5% acrylamide/0.087% *NN'*-methylenebisacrylamide or 15% acrylamide/0.087% *NN'*-methylenebisacrylamide [23]. Some gels were stained for protein with Commassie Blue R250; from others, proteins were transferred by electroblotting onto Immobilon P (Millipore) for Western analysis. Blots were first stained with Coomassie Blue R250, protein bands located, and then fully destained with methanol. Non-specific binding was blocked with 5% (v/v) newborn calf serum and 1% (w/v) BSA dissolved in 7.5% (w/v) glycine. The blot was then incubated for 1–2 h with PBS containing 0.1% Tween 20, 1% newborn calf serum and primary antibody, followed by incubation for 1 h with anti-rabbit peroxidase conjugate (Sigma product A-6154, diluted 1:2000). Visualization was achieved with enhanced chemiluminescence detection (Amersham). The total absorbance of Coomassie Blue-stained bands on SDS gels was measured with the Whole Band Analysis Package on a Millipore Bioimage instrument.

Protein chemistry

Protein concentrations were routinely determined with the Biorad dye binding assay. The assay was standardized with samples of full-length NOS of known protein concentration (determined by amino acid analysis). N-terminal sequencing was performed from blots on Immobilon P. Surface probability was calculated with the PeptideStructure option of the GCG package (Genetics Computer Group Inc., Madison, WI, U.S.A.), which is based on the method of Emini et al. [24].

Recombinant nNOS

Rat brain NOS was expressed in the baculovirus/insect cell system essentially as previously described [25], except that the medium was supplemented with 5 μg/ml haemin. Monolayers of infected cells were briefly washed with PBS at room temperature and then scraped off in the minimum volume of ice-cold 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA. The suspension was frozen rapidly and stored at −70 °C. All subsequent operations were at 4 °C. For purification, the suspension was thawed, sonicated for 5 s and centrifuged at 150000 ***g*** for 60 min. The supernatant was chromatographed on a column of 2′,5′-ADP-Sepharose (11 cm × 2.6 cm; Pharmacia), equilibrated with 10 mM Tris/HCl, pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 μM FAD, 1 μM FMN, 4 μM tetrahydrobiopterin, 1 mM arginine and 1 mM dithiothreitol (purification buffer). NOS was eluted with the same buffer supplemented with 10 mM NADPH and 6 μM tetrahydrobiopterin. NOS-containing fractions were pooled, concentrated in a stirred Amicon ultrafiltration cell (PM10 membrane) then further concentrated by precipitation with 78% saturated ammonium sulphate. The precipitated protein was dissolved in purification buffer (1.3 ml containing 24 mg of protein), applied to a column of Superdex 200 (Pharmacia; prep. grade 60 cm × 1.6 cm) and eluted with purification buffer supplemented with 50 mM NaCl. The NOS-containing fractions were pooled and concentrated in the Amicon cell to about 5 mg/ml. The yield from 1.2×10^9 cells was about 16 mg of NOS.

Limited proteolysis

Purified NOS protein (5 mg/ml protein) in purification buffer was incubated at 4 °C with either trypsin (tosylphenylalanylchloromethane-treated Sigma type XIII) or chymotrypsin (tosyllysylchloromethane-treated Sigma type VII). Ratios of protease to NOS between 1:500 and 1:5000 (w/w) were varied in different experiments. Samples were removed at intervals for analysis. The samples from incubations with trypsin were first added to a solution containing an excess of soybean trypsin inhibitor (Sigma product T-9003).

NOS (3 mg) was digested with 8 μg of trypsin in a volume of 800 μl for 2 h at 4 °C to obtain purified products of trypsin digestion. The reaction was stopped by addition of soybean trypsin inhibitor. The mixture was applied to a column of 2′,5′-ADP-Sepharose (1 ml volume) in 50 mM Tris/HCl, pH 7.5, containing 0.1 mM dithiothreitol. Unbound protein was removed

by washing with the same buffer, and the reductase domain was specifically eluted with buffer supplemented with 12 mM NADPH. The proteins that did not bind, and the proteins that were eluted with NADPH, were both further purified to near homogeneity by separate chromatography runs on a column of MonoQ (Pharmacia; 1 ml volume). The column was eluted with a gradient of 0–500 mM NaCl in 50 mM Tris/HCl, pH 7.5, containing 0.1 mM dithiothreitol.

Enzymic activities

NO synthase activity was measured by haemoglobin oxidation by using a Shimadzu dual-wavelength spectrophotometer at 37 °C. The assay mixture consisted of 50 mM Tris/HCl, pH 7.5, 0.2 mM $CaCl_2$, 0.1 mM dithiothreitol, 3 μM oxyhaemoglobin, 20 μM NADPH, 100 μM L-arginine, 10 μM tetrahydrobiopterin, 0.35 μM calmodulin, 1 μM FAD and 1μM FMN. The final addition was NADPH. The molar absorption coefficient ($\epsilon_{401-421}$) was taken to be 77 litre $\cdot$ mol^{-1} $\cdot$ cm^{-1}. Inclusion of 100 μM *S*-ethylisothiourea reduced the enzymic rate by over 99 %.

NADPH:cytochrome *c* reductase activity was measured at 550 nm (ϵ 18000 litre $\cdot$ mol^{-1} $\cdot$ cm^{-1}) at 37 °C in assay medium containing 50 mM Tris/HCl, pH 7.5, 0.2 mM $CaCl_2$, 1 mg/ml cytochrome *c* and 20 μM NADPH. Activity was measured either in the absence or presence of 0.35 μM calmodulin.

RESULTS

Limited proteolysis by trypsin

Full-length recombinant rat brain NOS was expressed in, and purified from, the baculovirus/insect system. The purified protein had a specific activity of 300–1000 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}, when assayed with the oxyhaemoglobin assay in the presence of calmodulin, FMN, FAD and tetrahydrobiopterin. Fresh preparations showed a single band by SDS/PAGE. On storage, bands of lower mobility were seen that seemed to be SDS-resistant aggregates or could represent covalently cross-linked protein. The protein bound [^{3}H]nitroarginine [26], and this binding was abolished by addition of excess arginine. The enzyme also had a high NADPH:cytochrome *c* reductase activity of about 10 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}. This activity was reduced to one-tenth when fresh enzyme was assayed in the absence of calmodulin. On freezing and thawing, the overall activity declined and the remaining activity was less calmodulin-dependent.

nNOS was incubated at 4 °C with trypsin in the presence of tetrahydrobiopterin and arginine. Samples were removed at intervals and proteolysis was stopped by addition of trypsin inhibitor. The samples were then analysed by SDS/PAGE (Figure 2), measurements of nitroarginine binding, NOS activity and NADPH:cytchrome *c* reductase activity (Figure 3), and by Western blotting with antibodies specific to defined regions of the NOS protein (Figure 4). Very small amounts of trypsin (1:5000) produced degradation of the full-length protein very rapidly with a half-life of approx. 3 min (Figures 2 and 3). NOS activity was lost with a significantly longer half-life (approx. 20 min), although a residual oxyhaemoglobin oxidizing rate of 5–15 % of the original remained even after prolonged digestion. This residual rate was confirmed to be due to NOS activity because it was inhibited by *S*-ethylisothiourea and a similar amount of residual activity was seen when the activity was measured by following the conversion of [^{3}H]arginine into [^{3}H]citrulline (results not shown). During this process no loss in nitroarginine binding or cytochrome *c* reductase activities was detected (Figure 3). Indeed, when measured in the absence of calmodulin, an increase in the reductase activity was observed. Proteolysis resulted in a loss in the ability of calmodulin to stimulate the cytochrome *c* reductase activity (Figure 3). SDS/PAGE revealed that three new bands were formed with apparent M_r values approx. 135000 (termed p135), 90000 (termed p90) and 80000 (termed p80). Western blotting showed that the p135 reacted with antibodies AB49, AB52-54, AB58, N31030 (Figure 4) as well as AB56 (results not shown), demonstrating that it contained at least from residue 270 up to the C-terminus, and hence the cleavage site was within the N-terminal 269 amino acids of NOS. This was confirmed by N-terminal sequencing, which showed that cleavage occurred at a tryptic site between residues Arg-220 and Gly-221. p80 reacted with AB56 (results not shown), AB58 and N31030 (Figure 4), but not with AB52-54, demonstrating that it contained the reductase region. In contrast,

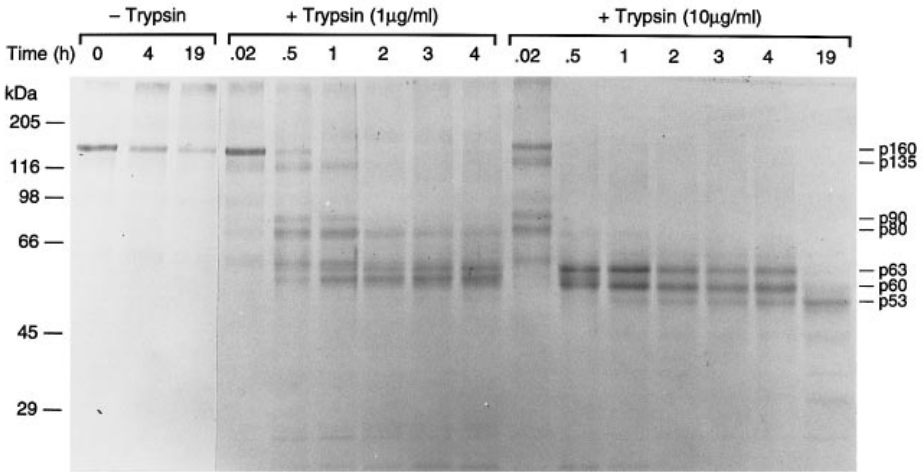

Figure 2 Limited proteolysis of nNOS by trypsin

nNOS (5 mg/ml protein) was incubated at 4 °C with either 1 or 10 μg/ml trypsin in the presence of 10 μM tetrahydrobiopterin and 100 μM arginine. At intervals, samples were analysed by SDS/PAGE. Protein was revealed with Coomassie Blue.

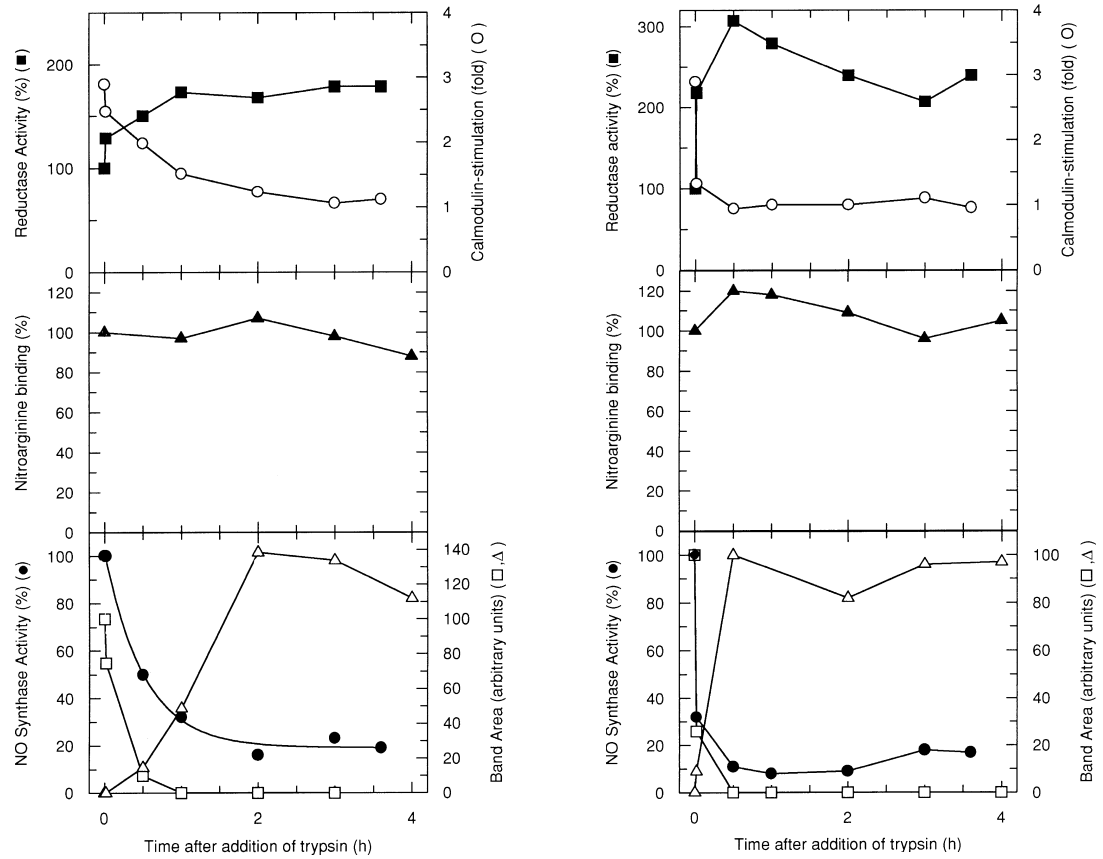

Figure 3 Effect of trypsin on functional properties of NOS

Samples from the experiment described in the legend to Figure 2 were analysed for their ability to bind [^{3}H]nitroarginine (▲) and to catalyse NO formation (●) and reduction of cytochrome *c* (○). Results are expressed as a percentage of the activities before the addition of trypsin. Cytochrome *c* reductase activity was measured both in the presence and absence of calmodulin; the activity shown is that in its absence. The amount of calmodulin stimulation is also plotted as a ratio of the activity in the presence of calmodulin to that measured in its absence (■). The integrated intensity of bands on Coomassie Blue-stained SDS/polyacrylamide gels showing the time courses of proteolysis are also plotted in the bottom panels. The bands quantified are p160 (□) and the sum of p63 and p60 (△). Data from experiments with 1 and 10 μg/ml of trypsin are shown in the left-hand and the right-hand panels respectively.

p90 reacted with AB49 and AB52-54, but not with AB 58 (Figure 4) or AB58 (results not shown), demonstrating that it was derived from the N-terminal half of NOS. The N-terminal sequence of p80 obtained from blots was AIGFXXLAE(V/A)VK, corresponding to residues 728 to 739 of NOS. Thus this protein was produced by tryptic cleavage between Arg-727 and Ala-728, within the calmodulin binding site. These data strongly suggest that trypsin cleaves NOS at residue 728, producing p80 and p90 proteins. These fragments seem to be similar to those seen by Sheta et al. [8]. When limited proteolysis with trypsin was performed in the presence of calmodulin the rate of cleavage at position 728 was reduced (results not shown), consistent with calmodulin's protecting this cleavage site. Simultaneously with this cleavage, another occurs at residue 221 to produce p135. The deduced trypsin cleavages are shown in Figure 5a.

Increasing time or increasing trypsin concentration (Figures 1–3) produced further cleavages, showing that the p90 and p80 species were merely transient species and did not represent the stable core domains of NOS. Prolonged trypsin treatment formed two stable species of apparent M_r approx. 63000 (p63) and approx. 60000 (p60). p63 reacted with AB58 (see below) and N31030, demonstrating that it was derived from the reductase region. The sequence of the N-terminus of p63 was K(N/S)XGDGPL, corresponding to residues 856–862 of NOS, consistent with its formation by cleavage at the trypsin site Arg-855/Lys-856. p60 reacted on blots with AB49 and AB52–54 but not with any antibodies to the reductase region of NOS. Its N-terminus was Gly-221. This pattern of two major bands remained for several hours, although some minor changes were observed in the reductase-derived fragments. At early time points p63 reacted with AB58 raised against a peptide derived from the actual C-terminus, whereas with longer incubation time this reactivity was lost, coinciding with a very small increase in mobility, suggesting the cleavage of some amino acids from the C-terminus (see below). At this stage of proteolysis (i.e. after 4 h), the unresolved mixture still retained full nitroarginine binding and cytochrome *c* reductase activity (Figure 3); the latter was despite cleavage between the two motifs required for FMN binding situated around residue 760 and around residue 900.

A tryptic digest of nNOS, consisting largely of p63 and p60, was applied to a column of ADP-Sepharose. The unadsorbed material had a UV spectrum consistent with bound haem and was able to bind [^{3}H]nitroarginine, but did not have cytochrome reductase activity. SDS/PAGE of this material showed predominantly a band of M_r approx. 60000 (results not shown). N-terminal sequencing revealed three sequences. The major sequence was consistent with a fragment produced by cleavage between residues 220 and 221. However, another sequence

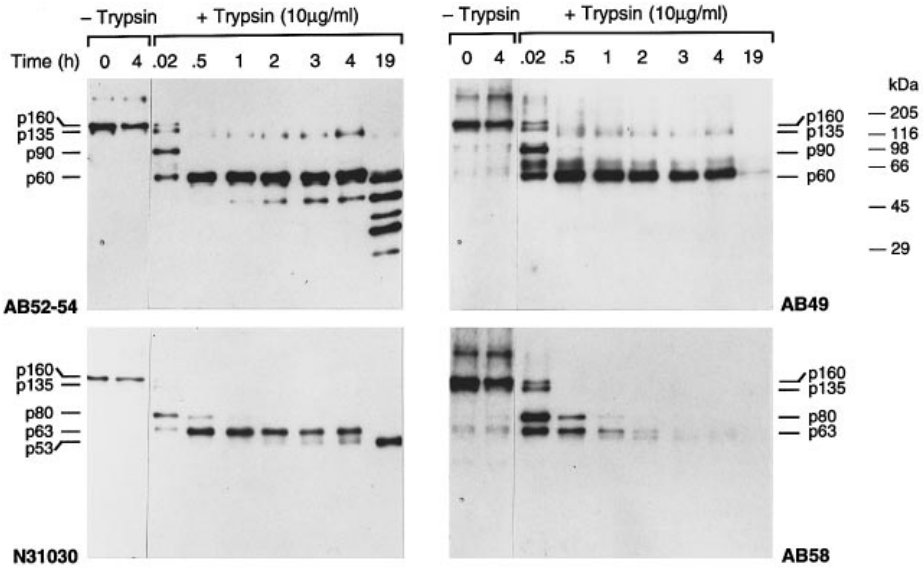

Figure 4 Identification of trypsin digestion products by immunodetection

Samples from the experiment described in the legend to Figure 2 with 10 μg/ml trypsin were analysed by immunoblotting. The four panels show detection with antibodies AB52-54, AB49, N31030 and AB58. Their epitopes are described in the Experimental section and are depicted in Figure 1.

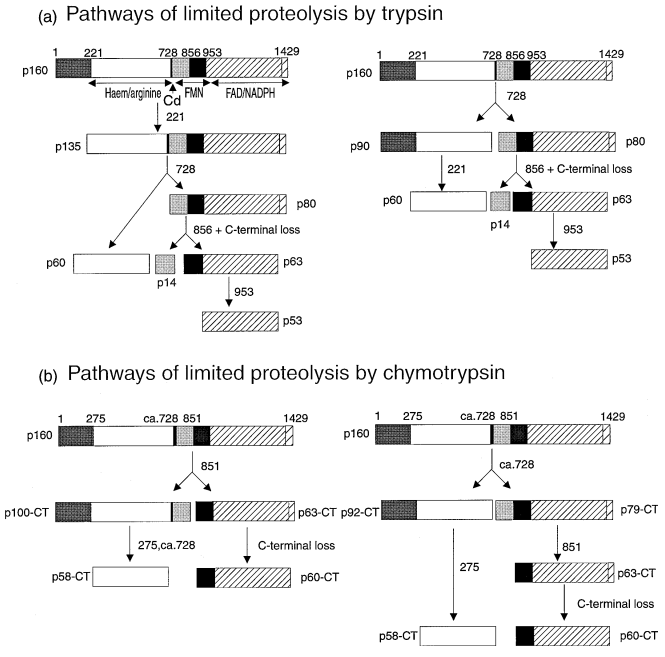

Figure 5 Schemes depicting proteolysis by trypsin and chymotrypsin

Our interpretation of the data obtained from limited proteolysis by trypsin and chymotrypsin. The numbers refer to the amino acid residue C-terminal of the proteolytic cleavage site. Abbreviation: ca., approximately.

SESIAFIEESKKDAXE was readily detected. This originated from a peptide produced by cleavage at a trypsin site between residues Arg-1409 and Ser-1410 close to the C-terminus of the protein. Another minor sequence corresponding to cleavage at the tryptic site between Lys-188 and Ala-189 was also observed.

After being washed, the column was eluted with NADPH producing a yellow eluate that did not bind [^{3}H]nitroarginine but retained cytochrome *c* reductase activity. It consisted predominantly of a protein of M_r approx. 63000 (p63) , but a band of M_r approx. 14000 (p14) was also present. N-terminal sequencing of the mixture revealed two main sequences: KSXXDGPDL, corresponding to residues 856–864, and LMGQAMAKRV, corresponding to residues 744–753 and possibly a minor sequence FNSV, i.e. residues 845–848. These would be produced by cleavages at the following tryptic sites: Arg-855/Lys-856, Lys-743/Leu-744 and Arg-844/Phe-845 respectively. This mixture was further chromatographed on a MonoQ column, resulting in co-elution of p63 and p14. p63 reacted with N31030 but not with AB55, whereas p14 reacted with AB55, showing that it was part of the FMN domain containing the FMN-phosphate binding motif. These data suggested that p14 had Leu-744 at its N-terminus and p63 had Lys-856 at its C-terminus. The co-elution of these two proteins on both ADP Sepharose and Mono Q suggested that they were associated non-covalently.

Prolonged trypsin treatment (e.g. overnight) resulted in the accumulation of a species of apparent M_r 53 000 (termed p53), which reacted with antibody N31030. The N-terminal sequence of this fragment was ANNSLISNDR, corresponding to residues 953–962 in the clone used in these studies [25]. The amino acid at position 953 has previously been published as being Pro rather than Ala [5]. We conclude that p53 has been produced by cleavage at the tryptic site Lys-952/Ala-953. After prolonged trypsin treatment, no single species accumulated but several bands, immunoreactive with AB52-54, of M_r approx. 35000–40000, were formed. None reacted with AB49. Two closely spaced bands with M_r approx. 35000 had sequences beginning at Thr-350 and Lys-344, presumably being formed by cleavage at the tryptic sites Arg-343/Lys-344 and Arg-349/Thr-350.

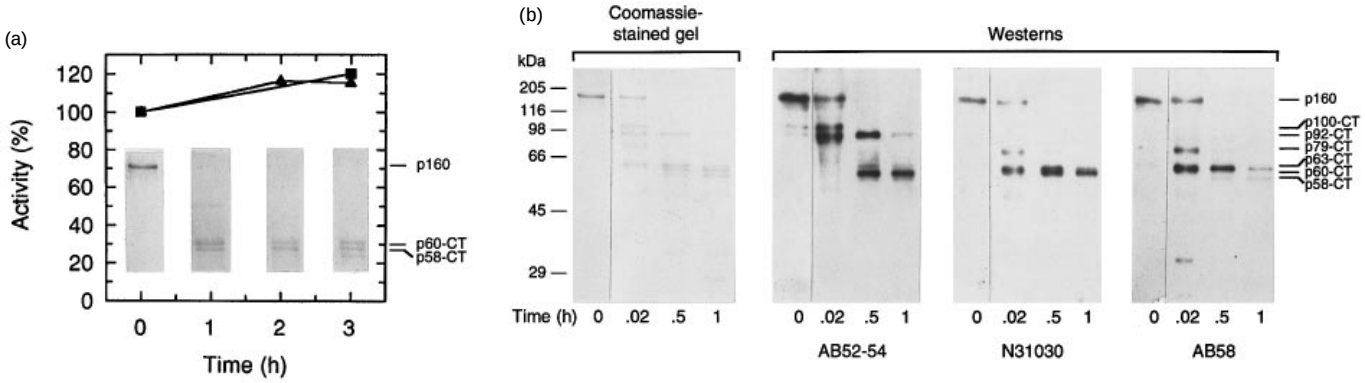

Figure 6 Limited proteolysis of NOS by chymotrypsin

nNOS (5 mg/ml protein) was incubated at 4 °C with 10 μg/ml chymotrypsin in the presence of 10 μM tetrahydrobiopterin and 100 μM arginine. At intervals, samples were analysed by SDS/PAGE and protein was revealed with Coomassie Blue. Gels are shown at times 0, 1, 2 and 3 h in (left-hand panel) and for shorter time periods in (right-hand panel). Samples were analysed for their ability to bind [^{3}H]nitroarginine (▲) and to catalyse reduction of cytochrome *c* (■) (left-hand panel). The latter activity was measured in the presence of calmodulin. Results are expressed as a percentage of the activities before addition of trypsin. Immunoblotting was performed with antibodies AB52-54, N31030 and AB58 (right-hand panel).

Limited proteolysis by chymotrypsin

Similar experiments to those described above, but replacing trypsin with chymotrypsin, were performed. After 3 h of incubation of NOS (5 mg/ml) with chymotrypsin (10 μg/ml) at 4 °C, both the nitroarginine binding and the cytochrome *c* reductase activity were the same as for the untreated protein (Figure 6). SDS/PAGE showed that two major protein bands were present, of apparent M_r approx. 60000 (p60-CT) and approx. 58000 (p58-CT) (Figure 6). These two proteins were of high stability as they were formed within 30 min and remained for at least 3 h. p60-CT, but not p58-CT, reacted with antibody N31030, demonstrating that p60-CT was derived from the reductase domain (Figure 6). p60-CT did not react with AB58, demonstrating that the C-terminus itself was lost. The N-terminal sequence of p60-CT was XDSRKSSGDGPDXRDN, corresponding to residues 852–867 of NOS, confirming that it was derived by cleavage at the chymotryptic site Tyr-850/Ser-851 within the FMN binding region. p58-CT, but not p60-CT, reacted with AB52-54, demonstrating that it originated from the N-terminal half of NOS. This was confirmed by the N-terminal sequence of p58-CT, the major sequence of which was SEKEQSPT, i.e. residues 275–284, with a minor sequence NNPY. i.e. residues 271–274. These fragments would be produced by cleavage at the chymotryptic sites Tyr-274/Ser-275 and Leu-270/Asn-271. Thus the overall pattern of digestion was similar to that seen with trypsin.

Examination of the time-course of proteolysis during production of p60-CT and p58-CT revealed subtle differences from that observed with trypsin. In particular, with chymotrypsin no protein of M_r between 110000 and that of full-length protein was produced, whereas with trypsin a protein of M_r approx. 135000 was a major early product. With chymotrypsin, bands of apparent M_r values approx. 100000 (p100-CT), 92000 (p92-CT) and 79000 (p79-CT) were formed initially. At later stages, a transient species of apparent M_r approx. 63000 (p63-CT) was formed. These proteins were characterized by Western blotting. Antibody N31030, reactive with the FAD/NADPH binding region, reacted with p79-CT and p63-CT. AB58 reacted with p63-CT and p79-CT. This showed that p79-CT and p63-CT were both derived from the reductase region and that p63-CT differed from p-60-CT in that the C-terminal epitope to AB58 was still present in the former. AB52-54 reacted with p100-CT, p-92-CT and a smaller fragment p58-CT. An interpretation of these data is shown in Figure 5b (see also Discussion).

DISCUSSION

Rapid proteolysis of rat brain NOS by trypsin occurred at residues 221 and 728. These cleavages occurred at broadly similar rates such that fragments p135, p63, p80 and p90 were produced simultaneously (Figure 5). The rate of cleavage at residue 728 was diminished by the presence of calmodulin, as previously reported [8]. Residue 728 is situated at the beginning of the calmodulin binding domain, which has been identifed between residues 725 and 754 [6,7]. Alignments of the protein sequences of nNOS, iNOS and eNOS show that the cleavage site at residue 221 of nNOS corresponds closely with the N-termini of eNOS and iNOS. Cleavage here results in the loss of the 220 amino acid N-terminal extension in nNOS containing the Discs-large Homologous Region [12]. The fragments p135, p80 and p90 seem to be the same as the major fragments characterized by Sheta et al. [8] although several other major bands were also apparent in their results, in particular an N-terminal-derived protein of M_r approx. 63000, which might correspond to our p60.

The experiments on proteolysis by Sheta et al. [8] were terminated at a stage when substantial amounts of undigested NOS were still present. In the experiments described here, proteolysis was allowed to continue until a limit digest was obtained. We found that the p135, p80 and p90 proteins were mere transients, and that three quite stable proteins were produced: p63, p60 and p14 (Figure 5). Significantly this mixture had the same ability to bind nitroarginine and the same, or higher, cytochrome reductase activity as the uncleaved full-length NOS (Figure 3), demonstrating that the proteolysis was dissecting the protein into functional regions.

During proteolysis, NOS activity was lost. The rate of decrease in activity was slower than the rate of loss of the full-length p160 band. Instead it correlated better with the loss of both the p160 and p135 bands, suggesting that the p135 protein maintained NOS activity. Interestingly, the fully proteolysed NOS, which contained no detectable p160 or p135, still catalysed NOS activity

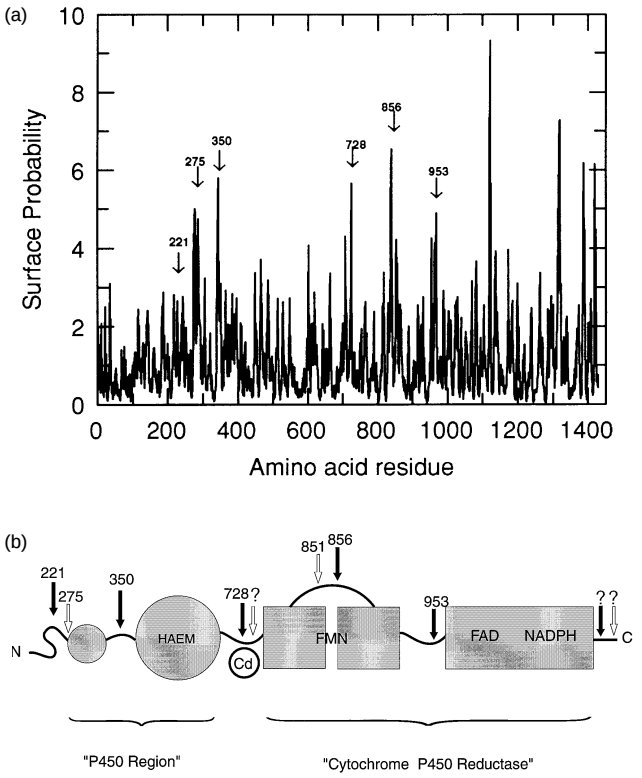

Figure 7 Domain structure of NOS

Top: the calculated surface probability of residues of nNOS: the arrows indicate the major tryptic and chymotryptic cleavage sites. Bottom: a model for the domain structure of nNOS based on these limited proteolysis studies: the arrows indicate sites of limited proteolysis by trypsin (black arrows) and chymotrypsin (white arrows). The sequence location of the proteolytic cleavage is noted above the arrow. The arrows with a question mark above them indicate cleavages producing fragments whose N-termini have not been precisely defined but for which the location of the cleavage has been deduced by reactivity with antibodies, the apparent M_r on SDS/polyacrylamide gels and the existence of other positively identified fragments.

at 5–15% of the rate of untreated NOS. This indicates that the isolated domains are able to reconstitute, albeit poorly, overall enzymic activity.

p60 corresponds to the N-terminal half of NOS after removal of residues 1–220 and the calmodulin-binding domain. We have not yet obtained its C-terminal sequence but its apparent M_r suggests that its C-terminus is close to residue 727. This protein was partly purified by chromatography on ADP-Sepharose, to which it did not bind, and on MonoQ. The protein contains bound haem and binds nitroarginine. These data strongly suggested that p60 contained the haem and arginine binding sites. This has been confirmed by expressing fragment 221–724 in *Escherichia coli* (A. Boyhan, D. Smith and P. N. Lowe, unpublished work).

p63 and p14 co-purified on ADP-Sepharose and MonoQ chromatography, but ran as discrete bands on SDS/polyacrylamide gels, indicating that they were in the form of a non-covalent complex. Immunodetection and sequencing suggested that p63 and p14 combined comprise the whole of the NADPH:cytochrome *P*-450 reductase region, and that they have been produced from p80 by a primary cleavage at residue 856 (Figure 5). Secondary proteolysis occurred at the C-terminus of p63 resulting in the loss of the last 23 amino acids, and also at the N-terminus of p14 resulting in its N-terminus being residue 744. Cleavage at residue 744 is at the most C-terminal of the trypsin sites within the calmodulin-binding peptide, and is close to the beginning of a region in NOS with strong similarity to the FMN-phosphate binding region of flavodoxins and of cytochrome *P*-450 reductase [27–29]. Residue 856 is situated in a region of NOS between the FMN-phosphate and FMN-isoalloxazine ring binding motifs. Specifically it is located in a stretch of about 40 amino acids with no similarity to other FMN-containing proteins and is between residues Phe-809 (analogous to Tyr-140 of human *P*-450 reductase) and Tyr-889 (analogous to Tyr-178 of human *P*-450 reductase), which are proposed to shield and stabilize the isoalloxazine ring [28,29]. This stretch of 40 amino acids is predicted to be exposed on the surface of the protein (Figure 7, top), and is present in both eNOS and nNOS, but is completely absent in iNOS. By comparison with the X-ray structure of flavodoxins [30,31], this region is an insertion in a loop connecting two β-strands. It is notable that this insertion is present only in eNOS and nNOS, both of which are regulated by Ca^{2+}/calmodulin, but is absent in both iNOS and in the BM3 ω-hydroxylase, neither of which is regulated in this way. It is tempting to speculate that it might be involved in the interaction between the FMN domain and the haem domain of NOS. p14 and p63 seem to be held together, after cleavage at residue 856, in a functional complex because it still retains cytochrome *c* reductase activity. The two proteins might be held together at least in part by the tightly bound FMN moiety, which could interact with both fragments.

The major stable proteins produced after limited proteolysis with chymotrypsin (p60-CT and p58-CT) are very similar in origin to the fragments p63 and p60 produced by trypsin cleavage. Both p60-CT and p60 are produced by cleavage within the insert region within the FMN domain. The sensitivity to both proteases confirms that the insert region is readily accessible to proteases. p58-CT represents the haem domain and is shorter than that produced by trypsin digestion, starting at residue 275 rather than 221. Notably, chymotryptic digestion of NOS to produce p58-CT did not result in a loss of nitroarginine binding activity (Figure 6, left-hand panel), suggesting that the arginine binding site is contained within the p58-CT fragment.

The rat brain cDNA clone used in this study [25] was identical in sequence to that published by Bredt et al. [5] with one exception: base 3205 was a G in the former but a C in the latter. This results in an amino acid difference: Pro-953 in the previously published sequence and Ala-953 in our clone. The N-terminal sequence of p53 verified that residue 953 was indeed alanine. Both the published mouse [32] and human [33–35] brain NOS sequences have alanine in the analogous positions, suggesting that the rat brain gene probably does encode alanine at residue 953.

We have compared the sites of limited proteolytic cleavage with the predictions for surface probability (Figure 7, top). It is noteworthy that, with the exception of the tryptic site at residue 221, most of the observed cleavage sites correspond quite closely to peaks in the predicted surface probability, supporting the suggestion that proteolysis is occurring at sites exposed in the native folded structure, and that these represent domain boundaries. It is interesting that the chymotryptic site at the N-terminus of p58-CT (residue 275) occurs at a region of high surface probability, whereas the trypsin site at residue 221 does not. It is therefore possible that residue 275 is much closer to the true start of the arginine-binding/haem domain than is residue 221, but perhaps there was no readily accessible trypsin cleavage site in that region.

The intron/exon boundaries for the human neuronal [35], endothelial [36] and inducible [37] NOS genes are generally

extremely similar. However, differences are seen in two places: first at the N-terminus where there is little or no sequence similarity between the isoforms and they have N-terminal extensions of different lengths, and secondly between residues 840 and 880 of nNOS, around the insertion in eNOS and nNOS relative to iNOS. In this region, a single exon encodes for the insertion present in eNOS and nNOS, and both proteases cut within this sequence. Chymotrypsin cleaves nNOS at residue 275, situated close to the first intron/exon boundary shared by all three isoforms; perhaps this is the start of the minimal haem/arginine binding domain.

The domain structure of the BM3 ω-hydroxylase [15,17–19,21] and of NADPH cytochrome P450 oxidoreductase [28,38] have been studied. Both of these enzymes, and NOS, contain an FMN and FAD-containing NADPH-dependent oxidoreductase, which is clearly similar in sequence [5,27]. Additionally BM-3, like NOS, is a haemoprotein, but there is little or no sequence similarity between them. A further difference between NOS and BM-3 is that only the former contains a tetrahydrobiopterin binding site. Limited proteolysis of BM-3 resulted in the production of two major proteins of M_r 66000 and 55000, which were derived from the reductase and haem domains respectively [15]. These proteins are of similar size to the equivalent regions derived from NOS. Extensive proteolysis resulted in further cleavage of the reductase into proteins of M_r 52000 [18] or 45000 [17], lacking the FMN domain. These proteins are analogous to the p53 fragment produced after extensive proteolysis of NOS, confirming the similarity in domain structure.

A model for the domain structure of NOS, based on these limited proteolysis studies and sequence similarities, is shown in Figure 7 (bottom). We expect that the definition of functional domains will allow the production of recombinant proteins with site-specific mutations to help in the precise identification of the binding sites for arginine, tetrahydrobiopterin and haem.

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