Cysteine-200 of human inducible nitric oxide synthase is essential for dimerization of haem domains and for binding of haem, nitroarginine and tetrahydrobiopterin

Rachael R. CUBBERLEY*†, Wendy K. ALDERTON*, Angela BOYHAN*, Ian G. CHARLES‡, Peter N. LOWE* and Robert W. OLD†§

*GlaxoWellcome, Medicines Research Centre, Gunnels Wood Road, Stevenage, Herts. SG1 2NY, U.K., †Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, U.K., and ‡The Cruciform Project, 140 Tottenham Court Road, London W1P 9LN, U.K.

Nitric oxide synthase (EC 1.14.13.39) is a homodimer. Limited proteolysis has previously shown that it consists of two major domains. The C-terminal or reductase domain binds FMN, FAD and NADPH. The N-terminal or oxygenase domain is known to bind arginine, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin) and haem. The exact residues of the inducible nitric oxide synthase (iNOS) protein involved in binding to these molecules have yet to be identified, although the haem moiety is known to be co-ordinated through a cysteine thiolate ligand. We have expressed two forms of the haem-binding domain of human iNOS (residues 1–504 and 59–504) in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins. The iNOS 1–504 and 59–504 fusion proteins bound similar amounts of haem, N^{ω}-nitro-L-arginine (nitroarginine) and tetrahydrobiopterin, showing that the first 58 residues are not required for binding these factors. Using site-directed mutagenesis we have mutated Cys-200, Cys-217, Cys-228, Cys-290, Cys-384 and Cys-457 to alanine residues within the iNOS 59–504 haem-binding domain. Mutation of Cys-200 resulted in a complete loss of haem, nitroarginine and tetrahydrobiopterin binding. Mutants of Cys-217, Cys-228, Cys-290, Cys-384 or Cys-457 showed no effect on the haem content of the fusion protein, no effect on the reduced CO spectral peak (444 nm) and were able to bind nitroarginine and tetrahydrobiopterin at levels equivalent to the wild-type fusion protein. After removal of the GST polypeptide, the wild-type iNOS 59–504 domain was dimeric, whereas the C200A mutant form was monomeric. When the mutated domains were incorporated into a reconstructed full-length iNOS protein expressed in *Xenopus* oocytes, only the Cys-200 mutant showed a loss of catalytic activity: all the other mutant iNOS proteins showed near wild-type enzymic activity. From this systematic approach we conclude that although Cys-217, Cys-228, Cys-290, Cys-384 and Cys-457 are conserved in all three NOS isoforms they are not essential for cofactor or substrate binding or for enzymic activity of iNOS, and that Cys-200 provides the proximal thiolate ligand for haem binding in human iNOS.

INTRODUCTION

Nitric oxide synthase [L-arginine,NADPH:oxygen oxidoreductase (nitric oxide forming), EC 1.14.13.39; NOS] is the enzyme responsible for the synthesis of nitric oxide (NO) from L-arginine, NADPH and O_2, generating L-citrulline as a co-product [1–4]. NO is involved in numerous physiological processes such as vasodilation, neuronal transmission and cytotoxic killing of bacteria and tumours (reviewed in [5–7]). Three different NOS isoforms have been identified: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). All three share a high degree of sequence and functional similarity and are encoded by different genes with different chromosomal localizations [8]. Calmodulin is required to facilitate electron transfer from the flavins within the C-terminal reductase domain to the haem moiety within the N-terminal oxygenase/haem domain of the enzyme [9,10]. The calmodulin-binding site of all three NO synthases is encoded on a single exon [11], and separates the reductase and oxygenase domains. The NO synthases are homodimers that contain binding sites for FAD, FMN and NADPH within their C-terminal portions [12,13]. Limited proteolysis studies on purified iNOS have located binding sites for arginine, (6*R*)-5,6,7,8-tetrahydro-L-biopterin (tetrahydrobiopterin) and haem within the oxygenase domain [14,15]. Tetrahydrobiopterin is essential for NOS activity although its precise role remains unclear. It has been shown to act as a stabilizing agent [16] and to be important in NOS dimerization [17,18]. The C-terminal domains of the NOS family demonstrate significant amino acid sequence similarity to NADPH:cytochrome *P*-450 reductase [14]. The NO synthases contain protoporphyrin IX haem, and their reduced CO spectrum shows a peak at approx. 450 nm, so that they are classified as cytochrome *P*-450 proteins. However, the N-terminal domain shows no clear sequence similarity to cytochrome *P*-450. The spectrum suggests that the NO synthases bind haem through a cysteine thiolate axial ligand [19–21]. The presence of FAD and FMN, and the sequence similarity to NADPH:cytochrome *P*-450 reductase, indicate that NO synthases are examples of 'catalytically self-sufficient' mammalian cytochrome *P*-450 enzymes, containing both a reductase and a haem domain similar to the fatty acid mono-oxygenase $P\text{-}450_{BM\text{-}3}$ isolated from *Bacillus megaterium* [22]. The bound haem functions directly in the oxidative conversion of L-arginine to nitric oxide and L-citrulline, and thus haem co-ordination is essential for enzymic activity [19,20]. Investigations on eNOS and nNOS have suggested that

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

§ To whom correspondence should be addressed.

the cysteine residue analogous to Cys-200 in iNOS (Cys-184 and Cys-415 respectively) provides the thiolate ligand to the haem [23–25], although none of the investigations confirmed that loss of haem binding was specifically caused by the cysteine mutation introduced; another possible reason for losing haem-binding ability could be alteration of the overall structure of the protein, thus blocking the access of haem to the haem-binding pocket. The NO synthases contain eight conserved cysteine residues within the haem domain. The purpose of the present study was to determine the precise cysteine residue involved in haem co-ordination in iNOS, and to determine the effects of mutating conserved cysteine residues on the binding of the substrate analogue N^{ω}-nitro-L-arginine (nitroarginine), on the binding of tetrahydrobiopterin and on dimerization.

EXPERIMENTAL

Materials

The cDNA encoding human chondrocyte iNOS was cloned in our laboratory [26]. N^{G}-Nitro-L-[2,3,4,5-^{3}H]arginine hydrochloride (specific radioactivity 51000 Ci/mol) was purchased from Amersham International. Research grade CO was obtained from BDH. pGEX-4T and glutathione–Sepharose were obtained from Pharmacia. Tetrahydrobiopterin dihydrochloride was purchased from Alexis Corp.

General methods

The *Escherichia coli* strains Novablue and BL21 (Novagen) were used for DNA manipulations and for protein expression respectively. Protein concentration was determined by the Bradford protein assay (Bio-Rad) with BSA as the standard. Proteins were separated by SDS/PAGE with a discontinuous buffer system [27] on a 12 % (w/v) separating gel. For immunoblotting, protein was transferred to Immobilon poly(vinylidene difluoride) membrane (Millipore). Non-specific binding sites were blocked with a 10 % (w/v) solution of reconstituted dried milk powder overnight at 4 °C. Primary antibody (see below) was added to the blot and incubated for 1 h at room temperature. Finally, peroxidase-conjugated rabbit anti-rat or goat anti-rabbit immunoglobulin was incubated with the blot. Bound immunocomplexes were detected by the enhanced chemiluminescence (ECL) method, as described by the manufacturer (Amersham International).

Construction of fusion genes encoding the iNOS haem domain

PCR was used to amplify DNA sequences encoding residues 1–504 or residues 59–504 of human chondrocyte iNOS. For amplification of the iNOS 1–504 region, primers were designed to incorporate *Sal*I/*Xho*I restriction sites. The N-terminal primer primer was 5′-ACCTGCTTTGTAAAGCCGTCGACATGGC-CTGTCCTTGG-3′; the C-terminal primer was 5-′AGACTTTC-AATGGAATCTCTCTCTCGAGTTAAAACTCCTCCTCGT-CCTGCCAGACATG-3′. For amplification of the iNOS 59–504 region, primers were designed so as to incorporate *Bgl*I/*Xba*I restriction sites. The N-terminal primer was 5′-CGGGAAGAT-CTCCCCTCGTGAGACGGGAAAGAAG-3′; the C-terminal primer was 5′-CGCCGCTCGAGTCAGAACTCCTCGTCCT-GCCAGACATGGGT-3′. For both the iNOS 1–504 and the iNOS 59–504 amplifications, the C-terminal primer was designed to incorporate a Glu-Glu-Phe tripeptide tag [which is recognized by the anti-tubulin antibody, YL1/2 (Serotec)] at the C-terminus of the haem domain. Reaction mixtures contained of 100 ng template DNA, each primer at 200 nM, 200 μM dNTP species, 1 × Pfu DNA polymerase buffer and 5 units of Pfu DNA polymerase (Stratagene) in 100 μl. The cycle conditions were 1 min at 94 °C, 2 min at 55 °C and 5 min at 72 °C, for 20 cycles. The amplified products were digested with *Sal*I/*Xho*I or *Bgl*I/*Xba*I, as appropriate, and ligated into *Sal*I/*Xho*I or *Bam*HI/*Xba*I-digested pGEX-4T expression vector. *E. coli* Novablue cells were transformed with the resultant ligation products. Recombinants were screened for DNA inserts of the correct size and confirmed by nucleotide sequencing. Fusion proteins were analysed by SDS/PAGE after induction of expression with 100 μM isopropyl β-D-thiogalactoside. Immunoblotting was also performed with anti-tubulin (YL1/2) and anti-NOS haem domain [28] antibodies.

Expression of iNOS haem domains

pGEX 1–504 and pGEX 59–504 were expressed in protease-deficient *E. coli* BL21 to minimize degradation of the protein. Cells were grown at 37 °C to a D_{600} of 0.3 in Terrific broth supplemented with 100 mg/ml ampicillin, 10 μM δ-aminolevulinate, 10 μM tetrahydrobiopterin and 5 μg/ml haemin (from a stock of 5 mg/ml in DMSO). The temperature was then reduced to 20 °C and the cells were equilibrated to this temperature for 1 h before induction with 100 μM isopropyl β-D-thiogalactoside. The cells were harvested by centrifugation after 16 h.

Purification of iNOS haem domains

All procedures were performed at 4 °C. The cell pellets were resuspended in ice-cold 10 mM Tris/HCl (pH 7.5)/50 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol/0.1 mM L-arginine/0.1 mM tetrahydrobiopterin/1 mM PMSF (buffer A). The cells were disrupted by sonication and centrifuged at 150000 ***g*** for 1 h. The supernatant was mixed with pre-equilibrated glutathione–Sepaharose for 1 h. The unbound protein was removed from the glutathione–Sepharose by washing with modified buffer A (NaCl increased to 300 mM). Purified protein was eluted with 5 mM reduced glutathione in modified buffer A, pH 8.0. Protein was quick-frozen in 0.1 ml aliquots. When purifying and expressing the seven cysteine mutants and wild-type iNOS fusion proteins, identical growth and purification conditions were used. Purification of all fusion proteins was performed simultaneously to minimize variation between samples.

Mutation of cysteine residues

The mutation of specific conserved cysteine residues within the iNOS haem domain was accomplished by overlap extension mutagenesis [29]. Oligonucleotide primers (Figure 1) were designed for the mutation of cysteine residues at positions 200, 217, 228, 290, 384 and 457 of human iNOS to alanine: Cys-200 was also mutated to histidine. As well as creating a single amino acid change, six of the seven mutations also incorporated a novel restriction site without altering any other amino acid residues. This enabled the rapid identification of recombinants. The cysteine mutations were confirmed by nucleotide sequencing the entire coding region.

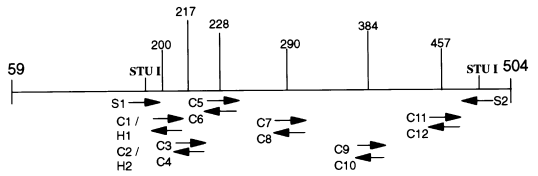
Determination of [^{3}H]nitroarginine binding

[^{3}H]Nitroarginine binding was determined essentially as previously described [15], except that 0.1 mM tetrahydrobiopterin was used and 500 mM NaCl was included in all buffers.

Determination of tetrahydrobiopterin binding

All purified protein was eluted in the presence of 0.1 mM tetrahydrobiopterin. To remove unbound tetrahydrobiopterin,

A



B

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Stu 1 S1     5' CACCAAGCAGGCCTGGCGCAATG 3'
Stu 1 S2     5' GGTTTTCCAGGCCTCTACCTG 3'
cys 200 C1   5' GCAATGCCCC***GCGCGC***CATTGGGAGGATCC 3'
cys 200 C2   5' GGATCCTCCCAATG***GCGCGC***GGGGCATTGC 3'
cys 217 C3   5' CGATGCCCGCAGC***GCTAGC***ACTGCCCGGGAA 3'
cys 217 C4   5' TTCCCGGGCAGT***GCTAGC***GCTGCGGGCATCG 3'
cys 228 C5   5' GTTTGAACACATC***GCGCGC***CACGTGCGTTAC 3'
cys 228 C6   5' GTAACGCACGTG***GCGCGC***GATGTGTTCAAAC 3'
cys 290 C7   5' GAATTCACTCA***GCTAGC***CATCGACCTGGGCTG 3'
cys 290 C8   5' CAGCCCAGGTCGATG***GCTAGC***TGAGTGAATTC 3'
cys 384 C9   5' AGTCCGGGACTTCGCTGACGTCCAGCGC 3'
cys 384 C10  5' GCGCTGGACGTCAGCGAAGTCCCGGACT 3'
cys 457 C11  5' TCCCGTGGGG***GCGCGC***CGGCAGACTGG 3'
cys 457 C12  5' CCAGTCTGCCG***GCGCGC***CCCCACGGGA 3'
his 200 H1   5' GCAATGCCCCAC***GCCACATTGGC***AGGATCC 3'
his 200 H1   5' GGATCCT***GCCAATGTGGC***GTGGGGCATTGC 3'
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Figure 1 Overview of the strategy used to create cysteine mutants

(**A**) The cysteine codons were mutated by overlap extension [29]. The PCR products incorporating the desired amino acid changes were reintroduced into the haem domain by using *Stu*I restriction sites. (**B**) The nucleotide sequences of the primers used to mutate the conserved cysteines are shown. Six of the seven mutants incorporated novel restriction sites; these are highlighted in italics. C200A, C228A and C457A contained a *Bss*HII site. C217A and C290A incorporated a *Nar*I site. C200H also incorporated a *Bgl*I restriction site.

the mutant proteins, wild-type proteins and a sample of the elution buffer were individually dialysed against ice-cold 10 mM Tris/HCl (pH 7.5)/300 mM NaCl/0.1 mM EDTA/1 mM dithiothreitol/0.1 mM L-arginine. The tetrahydrobiopterin in the samples was oxidized and analysed as described [30].

Determination of haem

The haem content of the purified fusion proteins was determined by the formation of the pyridine haemochrome after denaturation with alkali [31]. The protein (1 mg/ml), in 100 mM KOH and 30 % (v/v) pyridine, was reduced with a few grains of sodium dithionite. The spectrum was recorded from 500 to 600 nm and the absorbance difference between 600 and 556 nm was determined. The haem concentration was calculated by using an ϵ_{556} of 34700 $M^{-1} \cdot cm^{-1}$.

Reduced CO-binding spectra

The reduced CO spectra of iNOS haem domain were determined by bubbling CO gently through the solution of iNOS domain for 5–10 s. The NOS was then reduced by the addition of a few grains of sodium dithionite, after which the spectrum between 350 and 500 nm was recorded.

Non-denaturing gel electrophoresis

The GST polypeptide was removed from the iNOS 59–504 wild-type or C200A haem domains by thrombin cleavage of the GST fusion proteins bound to the glutathione–Sepharose matrix. (The pGEX constructs encoded a thrombin-sensitive linker polypeptide at the fusion between the GST and haem domain components of the fusion protein.) The oligomeric state of the haem domains was then analysed by electrophoresis under non-denaturing conditions, with an 8–25 % (w/v) polyacrylamide gradient gel (PhastSystem; Pharmacia). The migration properties of the native domains were revealed by immunoblotting and detection with anti NOS antibodies (AB52–54) [15,28].

Reconstruction of mutants into full-length iNOS

Full-length iNOS cDNA was manipulated by mutagenesis *in vitro* to remove two *Stu*I restriction sites situated towards the 3′ end of the coding sequence. The manipulated cDNA was then digested with *Stu*I to enable replacement of the wild-type restriction fragment with *Stu*I fragments bearing the individual cysteine mutations. The reconstructed full-length iNOS cDNA species were inserted into the vector pSP64T [32], and transcribed *in vitro* to generate synthetic mRNA.

Oocyte injection and expression

Expression of iNOS within oocytes was essentially as previously described [33]. *Xenopus laevis* oocytes were injected with about 50 ng of synthetic mRNA encoding wild-type or mutant full-length iNOS and incubated for 3 days at 18 °C in 50 μl (per five oocytes) of Barth X medium containing 2 mM arginine. NOS activity was determined by measuring nitrite production with the Griess reaction [34]. Translation of injected mRNA was confirmed by incubating groups of five oocytes at 18 °C in 50 μl Barth X medium containing 2 mM arginine and 15 μCi of [^{35}S]methionine. Homogenates of radiolabelled oocytes were analysed by SDS/PAGE; gels were dried and autoradiographed.

RESULTS AND DISCUSSION

Expression of the haem domain

Limited proteolysis of rat brain nNOS with chymotrypsin showed that cleavage at residue 275 (corresponding to residue 59 of human iNOS) resulted in a protein that retained its ability to bind arginine [15,30]. To examine the role of residues 1–58 of iNOS, human iNOS haem domains (residues 1–504 and 59–504) were successfully expressed in *E. coli* by using the pGEX-4T expression vector. To isolate fusion protein that was able to bind substantial amounts of cofactors and substrate, the purification buffers had to be supplemented with arginine and tetrahydrobiopterin (results not shown). The GST–iNOS fusion proteins were purified with glutathione–Sepharose. The purity and yield of GST–iNOS fusion protein (3 mg per litre of culture) purified from recombinant *E. coli* were similar for both haem domains [see Figure 2(B) for haem domain 59–504]. Densitometric analysis (results not shown) of the purified protein preparation indicated that the intact fusion proteins made up approx. 40 % of the sample by mass. The purified proteins were also analysed by immunoblotting. For each fusion protein the major band was recognized by YL1/2 antibody [30], which detects the tripeptide (EEF) tag inserted at the C-terminus [shown for haem domain 59–504 in Figure 2(C)]. The proteins of lower molecular mass evident in Figure 2(B) do not react with YL1/2 and so must be truncated at the C-terminus. For the purpose of calculating stoichiometry these were assumed to be inactive. The purified iNOS 1–504 fusion protein contained 0.2 mol/mol haem, as calculated by the formation of the pyridine haemochrome after denaturation of the protein with alkali, and 0.17 mol of tetrahydrobiopterin/mol of protein (Table 1). The fusion protein bound 0.1 mol of nitroarginine/mol of protein as estimated by the filter-binding assay. This binding was prevented

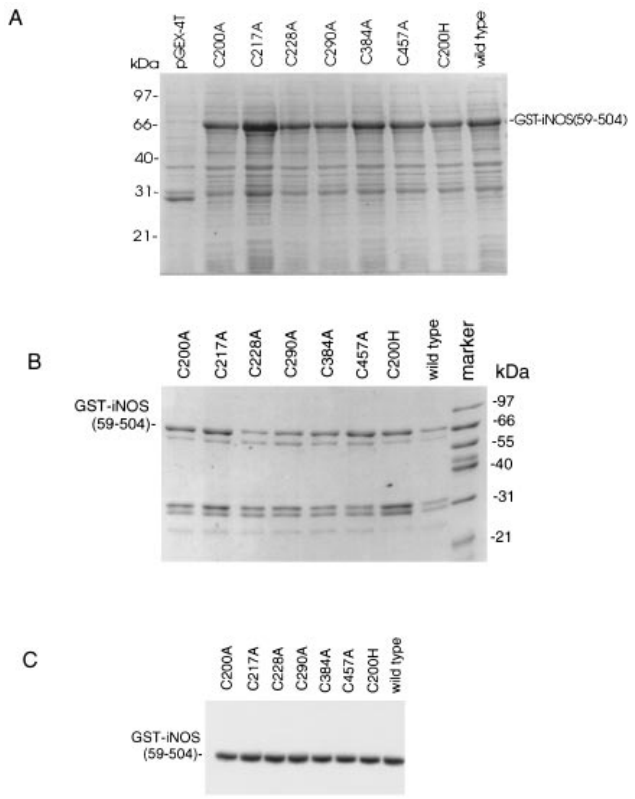

Figure 2 Analysis of the induced fusion proteins

(**A**) Bacteria expressing wild-type and mutant iNOS domains were induced with isopropyl β-D-thiogalactoside and analysed by SDS/PAGE. Protein was detected by Coomassie Blue staining. The positions of marker proteins for molecular mass are shown at the left. (**B**) SDS/PAGE analysis of the purified GST fusion proteins. Protein was detected by Coomassie Blue staining. The positions of marker proteins for molecular mass are shown at the right. (**C**) Immunoblot analysis of the purified fusion proteins. Immunoblotting was performed with the YL1/2 antibody, which recognizes the EEF tripeptide epitope used to tag the C-terminus of the fusion protein.

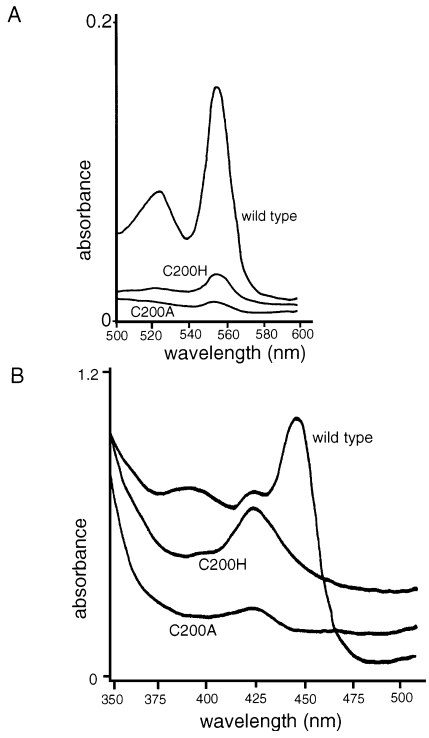

Figure 3 Pyridine haemochrome and reduced CO spectra of the wild-type and cysteine mutants

(**A**) The pyridine haemochrome spectra of the purified wild-type (iNOS 59–504) and the C200A and C200H mutant fusion proteins are shown. The spectra of the other mutant fusion proteins (C217A, C228A, C290A, C384A and C457A), which were indistinguishable from that of the wild-type fusion protein, are not shown. (**B**) The reduced CO spectra of the wild-type and the C200A and C200H mutant fusion proteins are shown. The reduced CO spectra of the other mutant fusion proteins (C217A, C228A, C290A, C384A and C457A), which were indistinguishable from that of the the wild-type fusion protein, are not shown.

by the addition of excess arginine. In this study we take the ability of the protein to bind nitroarginine (a slowly reversible inhibitor and substrate analogue) to be a measure of its ability to bind the natural substrate, arginine. Comparisons of fusion proteins containing residues 1–504 and residues 59–504 of iNOS showed no significant differences in expression levels in *E. coli* nor in the amounts of nitroarginine, haem or tetrahydrobiopterin bound, indicating that residues 1–58 are not required for binding these molecules. On reduction and binding of CO, the iNOS 1–504 (results not shown) and the iNOS 59–504 (Figure 3) fusion proteins exhibited a Soret absorption maximum at 444 nm, confirming that the haem is correctly co-ordinated through the thiolate ligand of cysteine. As the properties of iNOS 1–504 and iNOS 59–504 were indistinguishable, all subsequent experiments were performed on the shorter domain.

Mutation of cysteine residues within the haem domain, and analysis of the mutant fusion proteins

To determine which conserved cysteine residues were involved in haem co-ordination in iNOS, each of the cysteines at positions 200, 217, 228, 290, 384 and 457 within the 59–504 haem domain

Table 1 Binding of cofactor/substrate analogues to wild-type and mutant fusion proteins

The amounts of nitroarginine, haem and tetrahydrobiopterin that bound to each of the purified GST fusion proteins were measured as described in the Experimental section.

	Binding (mol of analogue/mol of monomer)								
Fusion protein ...	1–504	59–504	C200A	C217A	C228A	C290A	C384A	C457A	C200H
Nitroarginine	0.10	0.12	0.00	0.13	0.11	0.14	0.10	0.09	0.00
Haem	0.20	0.28	0.01	0.27	0.23	0.25	0.20	0.27	0.03
Tetrahydrobiopterin	0.17	0.18	0.00	0.17	0.18	0.22	0.13	0.15	0.00

was individually mutated to alanine by overlap extension mutagenesis (Figure 1) [29]. Cys-200 was also mutated to histidine. The other two conserved cysteine residues within this region of iNOS (Cys-110 and Cys-115) were not mutated because work within our laboratory has shown that the equivalent residues of nNOS are not essential for haem co-ordination [30].

The wild-type iNOS 59–504 and mutated haem domains were expressed in *E. coli* as GST fusion proteins (Figure 2A), and purified by affinity chromatography on glutathione–Sepharose. The wild-type and mutant fusion proteins were comparable in solubility, yield and purity (Figures 2B and 2C). This suggested that the mutations had no gross effect on the proteins.

The haem content of the purified fusion proteins was determined from the absorption peak at 556 nm of the pyridine haemochrome. The fusions containing the substitutions C217A, C228A, C290A, C384A and C457A all had significant and similar amounts of haem bound (Table 1) compared with the iNOS 59–504 wild-type fusion. The C200A mutant had negligible haem content, indicating that this cysteine residue is required for haem binding and suggesting that it is the cysteine residue involved in co-ordination of the haem. To investigate this further, the C200H mutant was isolated and studied because histidine has the ability to co-ordinate haem. The C200H mutation was found to decrease haem content severely, but the peak at 556 nm was significantly larger than for the C200A mutation (Figure 3A).

The reduced CO spectra (Figure 3B) of the purified wild-type iNOS fusion protein showed an absorption maximum at 444 nm, indicating that the haem is co-ordinated correctly to the proximal cysteine residue [19–21]. The reduced CO spectrum was determined for each of the cysteine mutants. The C217A, C228A, C290A, C384A and C457A fusion proteins all gave an absorption maximum of 444 nm, indicating the haem to be correctly co-ordinated to a cysteine residue in each of these mutants. As shown above, the C200A mutant had negligible levels of haem and hence no reduced CO peak was observed. When Cys-200 was mutated to histidine, the purified fusion protein contained a small amount of haem, and the reduced CO spectrum of this protein gave an absorption maximum of 422 nm. The spectral shift from 444 to 422 nm is consistent with haem co-ordination through the weaker imidazole ligand of histidine rather than the thiolate ligand of cysteine [35,36]. This observation is in agreement with those of McMillan and Masters [25], who showed low levels of haem binding to the equivalent cysteine residue when mutated to histidine in nNOS, although Richards and Marletta [24] were unable to detect haem binding to the equivalent mutated cysteine residue in nNOS purified from a baculovirus overexpression system.

The abilities of the cysteine mutants and wild-type fusion protein to bind nitroarginine were compared (Table 1). The mutants C217A, C228A, C290A, C384A and C457A all bound amounts of nitroarginine similar to that of the wild-type. Neither C200A nor C200H was able to bind nitroarginine (Table 1).

The amount of bound tetrahydrobiopterin was also determined for the wild-type and each of the mutant fusion proteins. The mutants C217A, C228A, C290A, C384A and C457A contained 0.13–0.22 mol of tetrahydrobiopterin/mol of protein, which is insignificantly different from that of wild-type iNOS 59–504 fusion protein (0.18 mol/mol). The C200A and C200H mutants did not contain any detectable tetrahydrobiopterin (Table 1).

It has been shown that iNOS is normally a homodimeric protein [17]. We therefore investigated whether the haem domain is itself capable of dimerization. First, the wild-type or C200A mutant haem domains were released from their fusion proteins by cleavage with thrombin. As measured by non-denaturing gradient electrophoresis, the apparent M_r of the wild-type domain

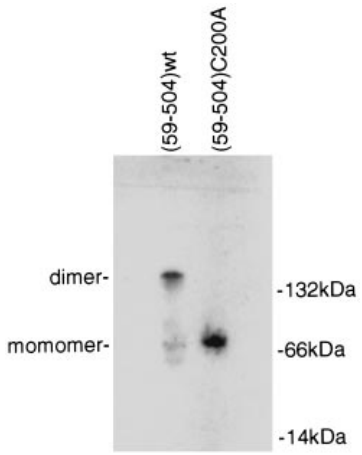

Figure 4 Non-denaturing gel electrophoresis of the wild-type (iNOS 59–504) and C200A mutant polypeptides

Purified wild-type (iNOS 59–504) and C200A mutant polypeptides were freed from GST by thrombin cleavage; 250 ng of each was subjected to electrophoresis on an 8–25% (w/v) polyacrylamide gradient gel. The wild-type and C200A mutant polypeptides were detected by immunoblotting with anti-NOS antibody. The positions of marker proteins for molecular mass are shown at the right.

(Figure 4) was similar to that of recombinant nNOS domain [30], strongly suggesting a homodimer. In contrast, C200A mutant haem domain ran with an apparent M_r half that of the wild-type protein, consistent with its being monomeric. The inability of the C200A mutant haem domain to form homodimers is most probably due to the loss of bound haem, arginine and tetrahydrobiopterin, as it is known that all three molecules are required for dimerization of intact NOS [17].

Expression of full-length wild-type and mutant iNOS

DNAs encoding the wild-type and mutated haem domains were reintroduced into full-length iNOS cDNA, which was transcribed *in vitro* to produce synthetic mRNA species. Synthetic mRNA species were individually injected into *Xenopus* oocytes. Translation of the mRNA species was confirmed by labelling with [^{35}S]methionine (Figure 5). The enzymic activity of the reconstructed cysteine mutants in iNOS was determined by measuring the accumulation of nitrite in the oocyte media. The wild-type iNOS produced approx. 23 μM nitrite per five oocytes. Although some variation in iNOS expression levels can be seen, with C217A translating most weakly, the complete absence of enzymic activity of C200A and C200H is clearly not due to poor expression. When the different amounts of iNOS protein expressed in the oocytes are taken into account, it is clear that C217A, C228A, C290A, C384A and C457A all had levels of enzyme activity similar to the that of wild-type protein. The oocytes expressed the C200A and C200H mutant iNOS proteins in amounts within the range of the other mutants, but showed no nitrite production (Figure 5). These results confirm that Cys-200 is essential to the enzymic activity of full-length iNOS. Although Cys-217, Cys-228, Cys-290, Cys-384 and Cys-457 are conserved in the three human NOS isoforms, and in iNOS homologues in other vertebrates including rat, mouse and chicken, these results show that these cysteine residues are not essential for the enzymic activity of iNOS.

Taken together, our results show that the mutation of Cys-200 to an alanine residue has dramatic effects on a number of

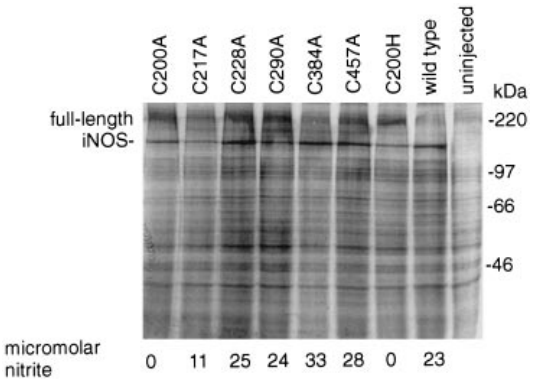

Figure 5 Determination of the level of the enzymic activity of wild-type and cysteine mutants expressed in oocytes

Wild-type and mutant full-length iNOS was expressed in *Xenopus* oocytes after injection with mRNA synthesized *in vitro*. Oocytes were radiolabelled with [^{35}S]methionine and the lysates were subjected to SDS/PAGE [10% (w/v) gel], followed by autoradiography. Expression of iNOS resulted in a labelled band consistent with the expected size of full-length iNOS at 130 kDa. In a parallel experiment, oocytes were injected and after 72 h the accumulation of nitrite in the oocyte culture medium was determined with the Griess reaction; nitrite concentrations (μM) are indicated under each lane. The positions of marker proteins for molecular mass are shown at the right.

properties of the haem domain itself and on the activity of the intact iNOS. Our results confirm proposals [23–25] that the corresponding cysteine residue in nNOS and eNOS is responsible for co-ordinating haem. Because it is possible that mutation of one cysteine residue might affect the overall protein structure to such an extent that the haem is prevented from co-ordinating with its target cysteine residue, the C200A mutant alone would not prove that this cysteine residue is the one responsible for haem co-ordination. However, we have also shown that mutation of the other conserved potential cysteine ligands of haem does not destroy haem binding and, importantly, does not destroy enzymic acivity. We therefore conclude that Cys-200 is responsible for haem co-ordination.

We thank H. Spence for assistance in preparing oligonucleotides, and M. Oxer for nucleotide sequence analysis. R.R.C. was supported by a CASE studentship jointly between the BBSRC and GlaxoWellcome.

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Received 9 August 1996/4 November 1996; accepted 6 November 1996