Expression in Yeast and Purification of Functional Macrophage Nitric Oxide Synthase. Evidence for Cysteine-194 As Iron Proximal Ligand

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ABSTRACT: Mouse macrophage NO-synthase (mNOS) was expressed in a unique yeast-based system by using a three-step procedure which allows yeast growth and NOS expression to be uncoupled. Despite cytotoxic effects related to mNOS expression, levels of catalytically active enzyme up to 0.5 mg of protein per 5 L of culture was obtained after purification. Its electrophoretic, spectroscopic [$\lambda_{max} = 446$ nm for its Fe(II)−CO complex], and catalytic properties were similar to those previously reported for mNOS purified from macrophages. Recombinant mNOS catalyzed the NADPH-dependent oxidation of L-arginine to citrulline ($K_m = 7 \pm 3$ μM) as well as the reduction of cytochrome *C* by NADPH [$K_m = 34 \pm 8$ μM and $V_m = 25 \pm 5$ μmol min^{-1} (mg of protein^{-1})]. Two mutants of mNOS in which Cys 194 was replaced with either serine or histidine were constructed and expressed in the same yeast strain at a level higher than that of the wild type protein, as they appear less toxic for the host. Both mutants exhibited electrophoretic properties and activities toward cytochrome *C* reduction identical to those of wild type NOS. However, they were unable to catalyze the oxidation of L-arginine to citrulline and did not appear to bind heme (no appearance of peaks around 400 and 446 nm for the resting enzyme and its CO complex, respectively, in visible spectroscopy). These data provide the first experimental evidence in favor of previous suggestions that Cys 194 was the proximal iron ligand of mouse mNOS.

The nitric oxide synthases (NOSs, EC 1.14.13.39)[1] are a family of heme-thiolate proteins which catalyze the five-electron oxidation of L-arginine to nitric oxide (•NO) and L-citrulline (Moncada et al., 1991; Stuehr & Griffith, 1992; Marletta, 1993). This transformation takes place in at least two distinct steps, given that N^{ω}-hydroxy-L-arginine has been shown to be a chemically- and kinetically-competent intermediate (Stuehr et al., 1991; Pufahl et al., 1992; Klatt et al., 1993). The NOSs have received a great deal of attention within the last eight years since •NO has been shown to mediate a diverse array of physiological responses. Among various other processes, •NO has been implicated in long-term potentiation and long-term depression, the stimulation and relaxation of gut and smooth muscle, vasodilation and platelet aggregation, and the immune system-related defense against tumor cells and invading intracellular microorganisms (Moncada et al., 1991; Schmidt & Walter, 1994).

Three isoforms of NOS are generally recognized, and are distinguished by their patterns of regulation as well as physical properties which influence their localization (Stuehr & Griffith, 1992; Marletta, 1993; Knowles & Moncada, 1994; Nathan & Xie, 1994). Isoform I (nNOS), of which the prototype is a 160-kDa protein isolated from rat neuronal tissue, is mainly cytosolic. It is constitutively expressed; however, its activity is regulated by calmodulin as well as changes in the intracellular concentration of Ca^{2+}. Isoform III (eNOS), which is found in endothelial cells, is constitutively expressed and regulated analogously to isoform I, but is largely membranal. eNOS, which is ∼130 kDa in size, contains a consensus sequence (MGXXXS) for N-myristoylation, and site-directed mutagenesis of the conserved glycine (Gly 2) gives rise to a cytosolic protein (Busconi & Michel, 1994). Unlike isoforms I and III, isoform II (mNOS) is not regulated by intracellular changes in Ca^{2+} concentration. The prototype for this isoform is a ∼130 kDa protein found in macrophages and other immune system cells, whose expression is induced upon the cells' treatment with various mediators of inflammatory responses. Although sequence analysis reveals that mNOS contains a consensus sequence for calmodulin binding, the activity of the enzyme, which is comparable to that of nNOS in the presence of Ca^{2+} and calmodulin, is essentially unaltered upon the addition of calmodulin. This apparent paradox was addressed by Cho

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[1] Abbreviations: •NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal nitric oxide synthase; eNOS, endothelial nitric oxide synthase; mNOS, macrophage nitric oxide synthase; H_4B, 6(*R*)-5,6,7,8-tetrahydro-L-biopterin; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; BSA, bovine serum albumin; DE-52, diethylaminoethyl cellulose; HPLC, high-pressure liquid chromatography; DTT, dithiothreitol; PVDF, poly[(vinylidene difluoride)]; PMSF, phenylmethanesulfonyl fluoride; PCR, polymerase chain reaction; P450, cytochrome P450; dNTPs, deoxynucleoside triphosphates; NNA, N^{ω}-nitro-L-arginine; SGI, glucose-based yeast minimum media; SLI, galactose-based yeast minimum media; SGIA, glucose-based yeast minimum media with adenine; SLIA, galactose-based yeast minimum media with adenine.

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et al. (1992), in which they showed that calmodulin is bound tightly to mNOS as if it was more a subunit of the enzyme rather than an effector.

Despite differences in location and regulation, there appears to be a commonality among the NOSs with respect to their modes of catalysis. Sequence analysis of representative proteins from each of the three isoforms reveals that the identity between any two isoforms is 50%−60% and that the C-terminal ends of the enzymes are ∼60% homologous to cytochrome P450 reductase, which is the only mammalian enzyme known to contain FAD and FMN within the same polypeptide (Bredt et al., 1991; Lowenstein et al., 1992; Lyons et al., 1992; Xie et al., 1992; Griffith & Stuehr, 1995). In addition, all NOSs characterized to date require NADPH, O_2, tetrahydrobiopterin (H_4B), and heme for the synthesis of ·NO (Mayer et al., 1991; Stuehr & Griffith, 1992; Marletta, 1993). UV−visible (McMillan et al., 1992; Stuehr & Ikeda-Saito, 1992; White & Marletta, 1992), resonance Raman (Wang et al., 1993), and electron paramagnetic resonance (Stuehr & Ikeda-Saito, 1992) spectroscopies have shown that the heme is bound through a cysteinate proximal ligand. Moreover, the reduced Fe(II)−CO spectra of all NOSs show absorbance maxima between 440 and 450 nm (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992; McMillan et al., 1992), a signature for the cytochromes P450 and few other heme-thiolate proteins (Mansuy & Renaud, 1995). That the NOSs are cytochrome P450-type proteins is particularly interesting, given that the 3A subfamily of the cytochromes P450 have been shown to be particularly efficient catalysts for the second half of the NOS transformation, the production of ·NO and citrulline from N^{ω}-hydroxy-L-arginine (Boucher et al., 1992; Renaud et al., 1993).

Given the physiological importance of NOSs with regard to their *in vivo* roles, as well as the unique reaction that they catalyze, it is desirable to develop alternative expression systems to produce adequate quantities of enzyme for further study and characterization, as well as to probe structure/function relationships by site-directed mutagenesis. The three isoforms of NOS have been expressed in various eukaryotic cell lines (Griffith & Stuehr, 1995) which gave in general low levels of enzymes. More recently, nNOS and eNOS have been produced in baculovirus-infected insect cells with much higher expression levels (Harteneck et al., 1994; Richards & Marletta, 1994; Nakane et al., 1995; Riveros-Moreno et al., 1995; Seo et al., 1995; Venema et al., 1995). Actually, mNOS was markedly less well expressed than the two other isozymes in that system (Moss et al., 1995; Nakane et al., 1995). Quite recently, nNOS was produced in yeast (Black & Ortiz de Montellano, 1995) and *Escherichia coli* (Counts Gerber & Ortiz de Montellano, 1995; Roman et al., 1995) with excellent expression levels, as high as 25 mg of NOS per liter of culture. The problem of the heterologous expression of mNOS appears more difficult as this enzyme presents an added problem in that, as a result of calmodulin being tightly bound, the flow of electrons from NADPH to the heme is less well regulated (Cho et al., 1992; Abu-Soud et al., 1994; Matsuoka et al., 1994). This means that during the expression of mNOS, $O_2^{\bullet-}$, ·NO, and other products resulting from cascades involving these two species can be produced, all of which are potentially lethal to the cell. Herein we report the successful expression of murine macrophage NOS in a unique yeast-based system by using a three-step procedure which allows yeast growth and NOS expression to be uncoupled. In addition, using site-directed mutagenesis, we report the first experimental evidence in support of Cys 194 as the proximal heme ligand in mNOS.

EXPERIMENTAL PROCEDURES

Materials. Horse heart cytochrome *C* (Type IV) and *Taq* polymerase were purchased from Boehringer Mannheim. Bovine liver catalase (10 000−25 000 units/mg), bovine erythrocyte superoxide dismutase (2500−7500 units/mg), FMN, FAD, NADPH, adenosine 2′,5′-diphosphate agarose, HEPES, glucose, hemin chloride, horse heart myoglobin, bovine serum albumin (Fraction V), galactose, sorbitol, streptomycin sulfate, ammonium sulfate, benzamidine, pepstatin, leupeptin, PMSF, sodium metabisulfite, aprotinin, arginine, N^{ω}-nitro-L-arginine (NNA), N^{ω}-methyl-L-arginine (NMA), and calmodulin were all obtained from Sigma. Bacto yeast extract, peptone, casamino acids, and yeast nitrogen base were obtained from Difco laboratories. Dithiothreitol (DTT) was obtained from Fluka. Dowex 50WX8-400 ion-exchange resin was purchased from Aldrich. 6(*R*)-5,6,7,8-Tetrahydro-L-biopterin (H_4B) was purchased from Dr. B. Schircks Laboratories (Jona, Switzerland). (*guanido*-^{14}C)-L-arginine (specific activity, 40−60 mCi/mmol), [U-^{14}C]-L-arginine (specific activity, 300 mCi/mmol), and (*ureido*-^{14}C)-L-citrulline (specific activity 59 mCi/mmol) were all obtained from Dupont NEN. DE-52 anion-exchange resin was from Whatman, and Sephacryl S-300 gel-filtration media was obtained from Pharmacia. DNA restriction and modification enzymes were from New England Biolabs.

Standard Procedures. General UV−visible spectroscopy was performed at ambient temperature using a Kontron 940 spectrophotometer. SDS−polyacrylamide gel electrophoresis (SDS−PAGE) was carried out using an SE600 Vertical Slab Unit (Hoeffer Scientific Units) following the procedure of Laemmli (1970), and the gels (7.5%) were stained with Coomassie Brillant Blue R-250. Protein concentrations were determined by the Bradford protein assay, using BSA as the standard.

Yeast Strains and Plasmids. *Saccharomyces cerevisiae* strains W303-1B (MATa; *ade*2-1; *his*3-11,-15; *leu*2-3,112; *trp*1-1; *ura*3-1; *can*^{r}; cyr^{+}) and the W303-derived W(R)*fur*1 have been previously described (Kern et al., 1990; Truan et al., 1993). The 2 μm-based yeast expression vector pYeDP8 (V8) contains a *URA3* selection marker as well as an expression cassette consisting of a *GAL10-CYC1* promoter and *PGK* terminator sequences (Urban et al., 1990). Expression vector pYeDP60 (V60) is analogous to V8 except that it carries an *ADE2* selection marker, and pYeDP80 is analogous to V60 except that the *ADE2* marker is replaced by a *TRP1* marker. Plasmid pMacNOS which contains the murine macrophage NOS cDNA was kindly provided by Dr. J. M. Cunningham of Brigham and Women's Hospital (Boston, MA) (Lyons et al., 1992).

In Vitro Amplification and Cloning of cDNA Encoding mNOS. The entire open reading frame of the cDNA encoding mNOS was amplified by PCR using the N-terminal primer **1**, 5′-d(GGAGATCTATGGCTTGCCCCTGGAAGTTTCTC)-3′, and the C-terminal primer **2**, 5′-d(GCGGTACCTCAGAGCCTCGTGGCTTTGGGCTC)-3′. Primer **1** contained in addition to the first 24 nucleotides of the mNOS gene sequence, a *Bgl*II restriction site adjacent to the ATG start site. Primer **2** contained the last 24 nucleotides of the

mNOS sequence as well as a *Kpn*I site just downstream of the TGA stop codon. The PCR reaction contained the following in a final volume of 50 μL: 1 ng of plasmid pMacNOS, 1 μM of each primer, 0.2 mM of each dNTP, 5 μL of 10× PCR buffer (100 mM Tris-HCl, pH 9, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton 100) and 1 unit of *Taq* DNA polymerase. Amplification was carried out with the aid of a Hybaid thermocycler, and 25 cycles of the following program were run: 94 °C for 5 s, 50 °C for 1 min, and 74 °C for 3 min. The amplified DNA (3436 bp) was digested with *Bam*HI, and the resulting 596-bp N-terminal fragment was ligated into a pUC 19 vector which had been previously digested with *Hin*cII (which leaves flush ends) and *Bam*HI. This intermediate pUC/NOS construct was digested with *Bsm*I and *Hin*dIII, and the resulting 587-bp *Bsm*I/*Hin*dIII fragment was exchanged with a *Bsm*I/*Hin*dIII fragment from the original pMacNOS vector. This yielded plasmid pMacORF, which contained the entire NOS gene, flanked immediately upstream by a *Bgl*II restriction site and downstream by a *Kpn*I restriction site. Using these two restriction sites from plasmid pMacORF, the mNOS open reading frame was cloned into pYeDP8 to yield pMacNOS8. Expression vector pMacNOS60 was generated by homologous recombination in yeast upon co-transforming yeast strain W303 with the 5132-bp *Pvu*II/*Eco*RI fragment isolated from pYeDP60 and the *Xho*I-linearized pMacNOS8 expression vector (Ma et al., 1987; Pompon & Nicolas, 1989).

Yeast Transformation and Cell Culture. Transformation of yeast strain W303 by pYeDP8, pYeDP60, pMacNOS8, or pMacNOS60 was performed as described by Gietz et al. (1992). Transformed yeast were plated on SGI media [0.7% (w/v) yeast nitrogen base, 0.1% (w/v) Bacto casamino acids, 2% (w/v) glucose] or SLI media [SGI media in which glucose is replaced with 2% (w/v) galactose] containing adenine (20 mg/mL). In the case of plasmids pYeDP60 and pMacNOS60 adenine was omitted.

The pMacNOS60/W(R)*fur1* strain was constructed in the following manner. Strain pYeDP80/W(R)*fur1* (Urban et al., 1994a) was transformed with the pMacNOS60 shuttle vector and then plated on SGI media containing tryptophan (20 μg/mL). Individual colonies were chosen and submitted to several serial rounds of plating on tryptophan-containing medium until loss of the pYeDP80 helper plasmid was accomplished, as judged by their inability to grow on media lacking tryptophan.

Preparation of Site-Directed Mutants. Murine macrophage NOS mutants C194S and C194H were constructed in the following manner. The N-terminal portion of mNOS was amplified by PCR using primer **3**, 5′-d(C_{-100}CGCCAGGCGTGTATAGGCC_{-81})-3′, and a reverse primer **4**, 5′-d(C_{600}CACTGGATCCTGCCGATGTGGCGAGGGG_{572})3′ (C194H), or primer **3** and a reverse primer **5**, 5′-d(C_{600}CACTGGATCCTGCCGATGCTGCGAGGGG$_{572}$)-3′ (C194S). The reaction conditions were as described above for the amplification of the mNOS cDNA; however, the thermocycling protocol consisted of the following: 20 s at 90 °C, 90 s at 45 °C, and 60 s at 72 °C for 2 cycles, followed by 20 s at 90 °C, 90 s at 55 °C, and 60 s at 72 °C for 20 cycles. In each case, a 672-bp fragment containing the appropriate mutations was gel-purified and used as a forward primer in a second PCR reaction in combination with a reverse primer **6**, d(C_{960}ACAAGATCAGGAGGGATTT$_{941}$). Again, the conditions for the PCR reaction are described above; however, the thermocycling protocol consisted of 25 cycles of the following program: 20 s at 95 °C, 30 s at 65 °C, 30 s at 60 °C 30 s at 55 °C, 30 s at 50 °C, 30 s at 45 °C, and 2 min at 72 °C. A 1061-bp fragment (bp −100 to 960) was isolated and digested with *Sph*I and *Bam*HI and ligated into pMacNOS60 which had been similarly digested and purified from its insert. W(R)*fur1* strains containing the mutant pMacNOS60 plasmids were created as described above.

Purification of Murine Macrophage NOS. Precultures of yeast strain pMacNOS60/W(R)*fur*1 grown in SGI (per liter: 1 g of Bacto casamino acids, 7 g of yeast nitrogen base, 20 g of glucose, and 20 mg of L-tryptophan) were used to inoculate 4.8 L of yeast complete medium (per liter: 5 g of glucose, 10 g of Bacto yeast extract, and 10 g of Bacto peptone). Cultures were grown at 28−29 °C (doubling time = 2 h) in an RFI-150 incubator/shaker (INFORS/Bottmingen, Switzerland) with constant shaking (180 rpms) until the yeast reached late-log phase (12 h, OD_{600} = 3−4), which corresponded to a density of $\sim 7 \times 10^7$ cells/mL. Ethanol was added to a final concentration of 3% (vol/vol), and the yeast were grown ∼10 h (OD_{600} = 7) on this carbon source. Finally, a 30% (wt/vol) solution of galactose was added to a final concentration of 2%, while hemin chloride was added to a final concentration of 5 μg/mL. The induction and expression of NOS proceeded for 9 h.

Upon completion of the expression phase, the yeast were quickly cooled on ice and then pelleted at 7000*g* for 10 min. The pellet was resuspended in buffer A (100 mM Tris-HCl, pH 7.5, 0.6 M sorbitol, 5 mM EDTA, 5 μM H_4B, 5 μM FAD, 5 μM FMN, 1 mM L-arginine, 3 mM DTT, 1 mM PMSF, 10 μg of pepstatin/mL, 10 μg of leupeptin/mL, 10 μg of aprotinin/mL, and 5 mM benzamidine) at a concentration of 0.5 mL per gram of wet weight (typical wet weight, 3 g). Two volumes of glass beads (Braun, 0.45−0.5 mm diameter) were added to the cell suspension, and the resulting mixture was transferred to a centrifuge tube which was approximately 2−3 times the volume of the cell suspension/glass beads mixture. The yeast were then lysed mechanically by manually shaking the centrifuge tube at 4 °C for a period of 10 min. The beads were removed with the aid of a fritted funnel, and the resulting crude extract was readjusted to pH 7.5 by the dropwise addition of a 1 M solution of Tris base. Additional aliquots of pepstatin A, leupeptin, aprotinin, benzamidine, and PMSF were added, and the crude extract was centrifuged at 4 °C and 100 000*g* for 1 h. Streptomycin sulfate (10% in buffer A) was added dropwise over 10 min to the resulting supernatant with concomitant gentle stirring. The solution was stirred for an additional 20 min and then centrifuged for 30 min at 4 °C and 18 000*g*. The resulting supernatant was slowly brought to 50% saturation by the addition of solid ammonium sulfate over a period of 20 min. After being stirred for an additional 30 min, the precipitate was pelleted by centrifugation as described above. The pellet was dissolved in a minimal volume (∼5 mL) of buffer B (50 mM Tris-HCl, 10% glycerol, pH 7.5, 3 mM DTT, 5 μM FAD, 5 μM FMN, 5 μM H_4B, 1 mM L-arginine, 1 mM EDTA, and 2 mM benzamidine) and then desalted on a column of Sephadex G-25. Colored fractions were pooled and then loaded onto a 2-mL column of 2′,5′-ADP agarose at a flow rate of 0.5 mL/min. The column was washed with 50 mL of buffer B containing 0.6 M NaCl and then 30 mL of buffer B containing 1.1 mM $NADP^+$ and 3 mM malic

acid. Lastly, the column was washed with 20 mL of buffer C (50 mM Tris-HCl, pH 7.5, 3 mM DTT, 5 μM H_4B, and 10% glycerol) and then eluted with 7 mL of buffer C containing 20 mM NADPH. Fractions containing significant NOS activity were pooled and loaded onto a column (0.75 $\times$ 5 cm) of DE-52 previously equilibrated in buffer C. The column was washed with 5 mL of buffer C and then 5 mL of buffer C containing 100 mM NaCl. NOS was eluted with 5 mL of buffer C containing 250 mM NaCl. NOS was concentrated to less than 100 μL with a Centricon 50 ultrafiltration device and then subjected to gel-filtration chromatography on a column of Sephacryl S300 (1 $\times$ 48 cm) previously equilibrated in buffer D (100 mM Tris-HCl, pH 7.5, 5 mM DTT, 10% glycerol, 2 mM EDTA, 10 μM H_4B, 5 μM FAD, 5 μM FMN, and 1 mM L-arginine). The column was run at a flow rate of 0.1 mL/min, and fractions displaying a significant absorbance at 400 nm (or 280 nm) were pooled and concentrated by ultrafiltration in a Centricon 50. An equal volume of 40% glycerol was added, and the protein was quickly frozen in liquid nitrogen before being stored at -80 °C.

Immunoblot Analysis. For Western blot analysis, NOS was subjected to electrophoresis as described above, and then transferred to a nitrocellulose membrane using a Trans-Blot SD semidry transfer cell (Bio-Rad) according to the manufacturer's specifications. The membane was blocked with a solution of 5% nonfat dried milk (NFDM) and 1% Tween-20, in phosphate-buffered saline (PBS), and then probed with a polyclonal antibody (1:1000 dilution in the above blocking solution) derived from the calmodulin binding domain of human inducible NOS kindly provided by Dr. W. A. Schmalix (Institüt für Toxikologie und Umwelthygiene der Technischen Universität, München, Germany). After appropriate washing steps as described by the manufacturer, the filter was developed with a secondary swine anti-rabbit antibody conjugated to horseradish peroxidase, in combination with the chromogenic substrate diaminobenzidine.

Activity Determination. Nitric oxide synthase activity was measured by two different methods. The first method was by the time-dependent production of citrulline. Each assay included in a final volume of 500 μL 50 mM HEPES, pH 7.4, 3 mM DTT, 2 mM $CaCl_2$, 1 mM NADPH, 5 μM H_4B, 5 μM FAD, 5 μM FMN, 10 μg of calmodulin/mL, 100 μM (*guanido*-^{14}C)-L-arginine (specific activity, 6.5×10^6 to 1.1×10^8 cpm/μmol), and 10$-$30 pmol (25$-$75 nM) mNOS. A 100-μL aliquot was removed ($t = 0$), and the reaction was initiated by the addition of NOS (3$-$5 μL) to the reaction mixture. At various time intervals, subsequent aliquots of the same size were removed and added to 50 μL of 2% perchloric acid to quench the reaction. All reactions were subsequently reneutralized with 65 μL of 0.2 N KOH, and 800 μL of a solution containing 50 mM sodium acetate, pH 5.5, 1 mM citrulline, 2 mM EDTA, and 0.2 mM EGTA. The mixtures were applied to 1-mL Dowex 50WX8-400 ion-exchange columns, and then washed with 2 mL of H_2O. A 500-μL aliquot of the eluate was then subjected to scintillation counting. A control reaction containing radiolabeled citrulline (232 pmol, specific activity, 59 mCi/mmol) was carried out in the same manner to determine the efficiency of citrulline elution. To verify the nature of the products produced, incubations were often analyzed by HPLC (Spectra-Physics S2000) with on-line radioactivity detection (Berthold LB 510) using Quick Zint scintillation cocktail pumped in at a flow rate of 3 mL/min. The column (Zorbax SCX 300, 7 μm, 250 $\times$ 4.6 mm) was equilibrated in solvent A (0.02 M ammonium acetate, pH 2.5) and then washed with the same solvent for 7 min at a flow rate of 1 mL/min. Next, a 20-min gradient was applied from 0%$-$100% solvent B (0.1 M ammonium acetate, pH 3.3), with L-citrulline, L-NOHA, and L-arginine eluting at 4.3, 18.0, and 21.0 min, respectively.

The second method was by the formation of methemoglobin from the reaction of ·NO with oxyhemoglobin. Assays were carried out essentially as described by Hevel and Marletta (1994). Oxyhemoglobin was prepared by treating hemoglobin with dithionite and then removing the dithionite by gel-filtration using Sephadex G-25. The purity and concentration of the protein solution were determined spectrophotometrically (absorbance maximum, 415 nm, $\epsilon =$ 131 mM^{-1} cm^{-1}) (Murphy & Noack, 1994). The formation of methemoglobin was quantified by the time-dependent increase in absorbance at 401 nm ($\Delta\epsilon = 60\,000$ M^{-1} cm^{-1}) (Hevel & Marletta, 1994) using a Cary model 210 spectrophotometer thermostated at 37 °C.

Kinetic characterization of mNOS was carried out with the radioactive assay as described above using Dowex cation-exchange resin to separate the product from the starting material. All kinetic data reported are the averages of at least three time-dependent trials. For the determination of kinetic constants for substrate, L-arginine concentrations were varied from 0.4$-$20 μM, and values for K_m and V_{max} were obtained from Lineweaver$-$Burk plots.

Assays to monitor cytochrome *C* reductase activity contained the following in a final volume of 1 mL: 50 mM HEPES, pH 7.4, 1 mM EDTA, 1 mM NaCN, 40 μM cytochrome *C*, 100 μM NADPH, and the appropriate amounts of NOS. NaCN was used to inhibit cytochrome *C* oxidase which could be present as a contaminant from yeast mitochondria. All components of the assay except NOS were mixed and equilibrated at 25 °C. Upon the addition of NOS, the reduction of cytochrome *C* was measured by the time-dependent increase in absorbance at 550 nm ($\Delta\epsilon = 21\,000$ M^{-1} cm^{-1}) (Strobel & Dignam, 1978).

Analysis of the Ability of Mutants C194S and C194H to Effect Substrate Turnover. The reaction mixture was as described for assays of the wild type mNOS, except the total volume was 100 μL and [U-^{14}C]-L-arginine (specific activity, 7×10^7 cpm/μmol) was employed. The reactions were started by the addition of NOS ($\sim$0.25 mg of each purified mutant or $\sim$0.50 mg of each of the mutant crude extracts) and proceeded for 1 h at 37 °C. The reactions were then analyzed directly by HPLC with on-line radioactivity detection as described above.

Spectral Characterization of mNOS. Macrophage NOS was exchanged into buffer containing 50 mM Tris-HCl, pH 7.5, and 10% glycerol. A couple of grains of dithionite were added (500 μL, 1 μM mNOS), and then the solution was equally distributed between the sample and reference cuvettes. After recording a base line, CO was bubbled at the surface of the sample cuvette for 5 s. A difference spectrum was then immediately recorded from 400 to 500 nm at a scan rate of 100 nm per minute.

RESULTS

Cloning and Expression in Yeast. Our choice of a yeast-based system was governed by its feasibility of use and low

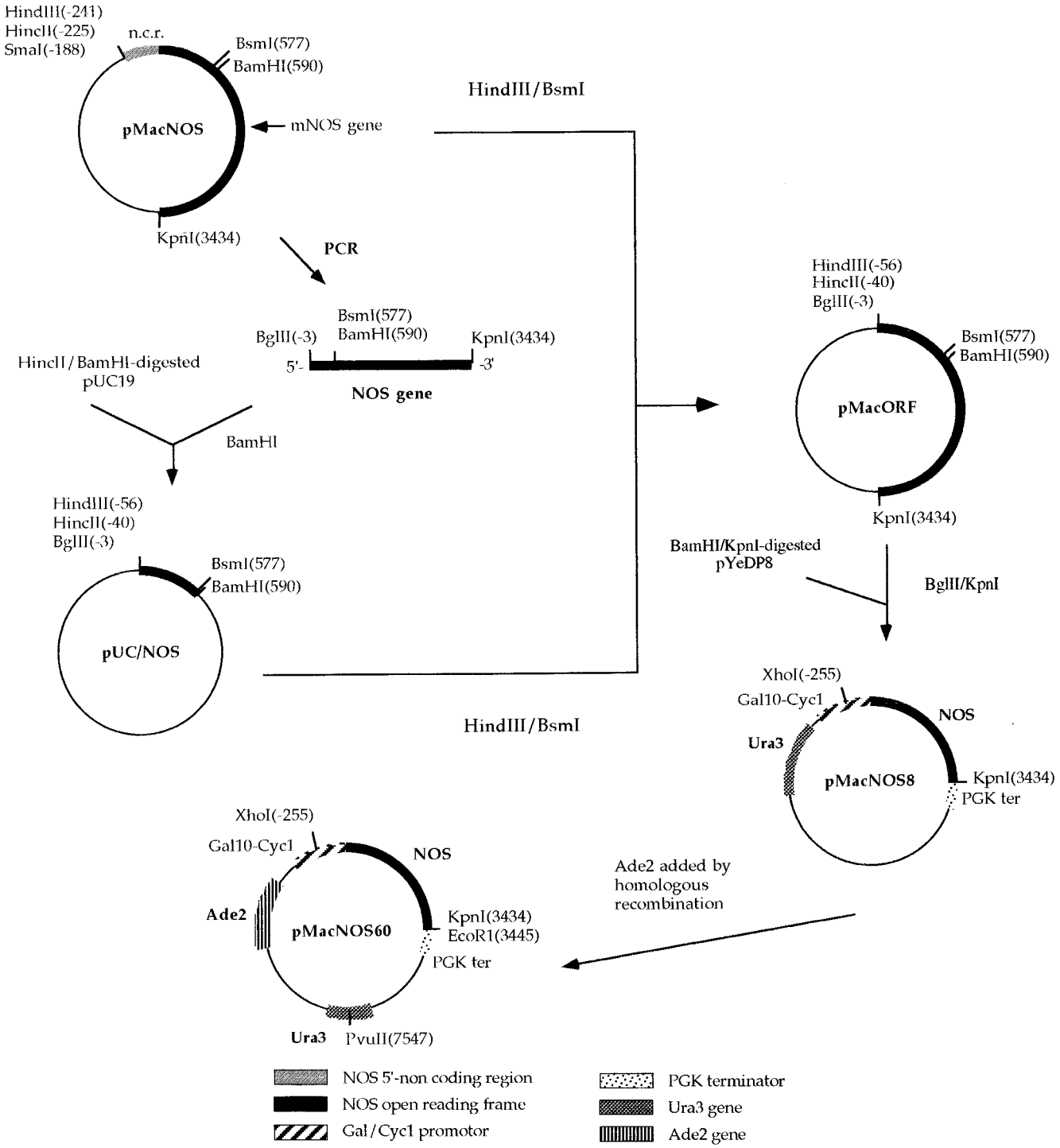

FIGURE 1: Construction of the yeast expression vector pMacNOS60. The full-length NOS cDNA was PCR amplified to eliminate the 5′ noncoding region and the 5′ portion spanning the ATG and the *Bam*H1 site was cloned into a puc vector and sequenced. A *Hin*dIII/*Bsm*I DNA fragment of this new 5′ region was used to replace the original *Hin*dIII/*Bsm*I DNA fragment, generating a *Bgl*II/*Kpn*I cassette containing the mMacNOS open reading frame in its original cloning plasmid. The *Bgl*II/*Kpn*I-digested mMacNOS cDNA was then placed under the transcriptional control of a yeast inducible *GAL10-CYC1* promotor upon subcloning into a *Bam*HI/*Kpn*I-digested pYeDP8 yeast shuttle vector. Finally an additional *ADE2* marker was added to the vector upon homologous recombination between the pMacNOS8 vector and an homologous portion of a yeast vector containing the *ADE2* gene to generate the pMacNOS60 expression vector. Restriction sites of interest are shown on the plasmid maps with their respective cleaving positions under brackets.

cost as compared to commercial systems such as baculovirus-infected insect cells as well as the success in using yeast for the expression of various cytochromes P450 (Oeda et al., 1985; Guengerich et al., 1991; Peyronneau et al., 1992) and P450−P450 reductase fusion proteins (Shibata et al., 1990).

Taking into account the possible cytotoxic effects of expressed mNOS (see above), we chose to clone the cDNA for mNOS behind a *GAL10-CYC1* promoter, therefore allowing the production of mNOS to be induced only upon the addition of galactose to the growth media. Our cloning strategy is outlined in Figure 1. Since the presence of excess nucleotides upstream of the ATG start codon is known to be incompatible with efficient expression in *S. cerevisiae*, this stretch of DNA had to be deleted from the original mNOS cDNA (Cullin & Pompon, 1988; Pompon & Nicolas, 1989). This was accomplished using a PCR-based approach

in which the N-terminal primer carried a *Bgl*II restriction site at its 5′ end. Although the entire mNOS gene was amplified, only an N-terminal 594-bp fragment was used to create an intermediate pUC19/NOS construct. The *Hin*dIII/*Bsm*I fragment of the pUC19/NOS construct was then exchanged with a similarly-digested fragment from the original pMacNOS plasmid, giving rise to pMacORF which contained the entire mNOS gene flanked by *Bgl*II and *Kpn*I restriction sites. The entire mNOS gene was then excised using these two restriction sites and cloned into the expression vector pYeDP8, resulting in vector pMacNOS8. This vector carries the entire mNOS gene flanked by a *GAL10-CYC1* hybrid promoter and *PGK* termination sequences as well as a *URA3* selection marker and a yeast 2-μm origin of replication.

Initial studies on the expression of mNOS in yeast were carried out with pMacNOS8 transformed in yeast strain W303. Strain pMacNOS8/W303 was grown to mid-log phase in SGIA, a yeast minimal medium with glucose as the carbon source. Upon dilution of the exponentially-growing yeast into a galactose-containing minimal medium (SLIA) in order to allow both cell growth and mNOS induction to take place, yeast cell growth was strongly inhibited. The doubling time of 2.5 h obtained in SGIA became greater than 5 h upon switching to galactose as the sole carbon source. Moreover, yeast growth halted upon the attainment of a density of 2×10^6 cells mL^{-1} or 1 OD_{600}. Upon harvesting, no formation of [U-^{14}C]-L-citrulline from the correspondingly-labeled arginine (using HPLC with on-line radioactivity detection) was detected in the cytosols, microsomes, or mitochondria of these yeast (data not shown). In addition, no cytosolic cytochrome P450 reductase activity was detected that was above the level of that of the same yeast transformed with a control pYeDPV8 plasmid [specific activity, 0.5 nmol min^{-1} (mg of protein)$^{-1}$]. These results led us to postulate that under the given conditions the expression of mNOS was inhibiting yeast growth.

Expression of mNOS Using the fur System. The failure to successfully induce the expression of mNOS in yeast using classical technology necessitated a revision in our strategy. We chose to use a unique yeast expression system in combination with a three-step procedure which would allow yeast growth and mNOS expression to be uncoupled. The *fur* system allows expression to be carried out in enriched medium without consequent loss of the plasmid of interest (Urban et al., 1990; Truan et al., 1993). Yeast strain W(R)-*fur1* contains chromosomal mutations in the genes encoding *ura 3* (OMP decarboxylase) and *fur 1* (uracil phosphoribosyltransferase) and is thus unable to synthesize uracil or extract it from the growth medium. Thus, only yeast which harbor a plasmid encoding *ura 3* is viable. Since this strain is nonviable alone, it is maintained in the presence of a helper plasmid (pYeDP80) which encodes the *ura 3* gene. After transformation of the mNOS-containing plasmid (with *ade 2* and *ura* 3 as selection markers), the helper plasmid (with *ura 3* and *trp 1* as selection markers), was removed by serial plating on media containing tryptophan, but lacking uracil and adenine.

The yeast were first grown on glucose (in which the yeast grow anaerobically) until this sole carbon source was expended. Next, ethanol was added as the sole carbon source, which allowed the yeast to quickly and efficiently switch to aerobic growth. Finally, aerobic respiration was continued with the addition of galactose while simultaneously inducing the production of mNOS (Truan et al., 1993; Urban et al., 1994b). Since the *GAL10-CYC1* promoter is not functional under anaerobic growth, the addition of galactose immediately after growth on glucose was inefficient, as the yeast require too much time to switch to aerobic growth. Thus an intermediate growth period on ethanol was used to allow the yeast to convert to aerobic growth before induction of mNOS with galactose. Under these conditions, NOS and cytochrome *C* reductase activities were detected and reached maximum levels (~500 pmol of citrulline min^{-1} and 10 nmol of reduced cytochrome *C* min^{-1} per mg of crude extract) 9 h after the addition of galactose. Before the addition of galactose, no time-dependent production of citrulline was detected, while the level of cytochrome *C* reductase activity was measured to be less than 1 nmol min^{-1} mg^{-1}.

Table 1: Recombinant mNOS Purification Profile

step	volume (mL)	protein (mg)	specific activity[a] (nmol min^{-1} mg^{-1})	units (nmol min^{-1})
crude	180	2304	0.20[b]	460
G-25	50	640	0.47	300
2′-5′-ADP agarose	9	4.32	40	172
DE-52	2.6	1.3	86	112
S300	0.8	0.48	110	53

[a] Citrulline production was measured using the Dowex assay (as described in the experimental part) with 10-μL aliquots of each fraction. [b] Activity determined using HPLC analysis with on-line radioactivity detection, as the Dowex assay cannot be used for this crude lysate which contains other activities transforming L-arginine. These activities were lost upon ammonium sulfate precipitation.

Purification of mNOS. The purification of mNOS was carried out using modifications of previously established procedures (Baek et al., 1993). Upon lysing the yeast, cell debris were removed by centrifugation at 10 000*g*, and then the crude lysate was further clarified by centrifugation at 100 000*g* to remove the microsomes. Unlike mNOS isolated directly from macrophage cell lines, or that expressed in baculovirus, the mNOS in our yeast crude lysate did not bind efficiently to either adenosine 2′,5′-diphosphate agarose (Sigma) or adenosine 2′,5′-diphosphate Sepharose (Pharmacia). As a result, we have included a nucleic acid precipitation step before precipitating mNOS with $(NH_4)_2SO_4$. Upon removal of $(NH_4)_2SO_4$ by gel-filtration, mNOS bound to the affinity column with high efficiency. In fact, under the described conditions, NADPH concentrations of 20 mM were routinely needed to elute the protein. A typical purification profile is shown in Table 1. From 5 L of recombinant yeast culture 200−500 μg of mNOS of specific activity ~100 nmol min^{-1} mg^{-1} can be routinely isolated.

Characterization of Recombinant mNOS. mNOS runs at ~130 kDa on SDS−PAGE (Figure 2A) and is well recognized by an anti-inducible NOS antibody (data not shown). It is active for the oxidation of L-arginine to citrulline only in the presence of NADPH (Table 2), the formation of citrulline being linear as a function of time for at least 20 min (data not shown). As expected, addition of Ca^{2+} and calmodulin has little effect on the activity whereas classical inhibitors of NOS such as *S*-methylthiocitrulline and *N*-nitroarginine (NNA) strongly decrease its activity (Table 2). Figure 3 shows the effect of H_4B on the time course of mNOS turnover. At the initial time point (2 min), there is little difference between the reaction containing H_4B and that lacking H_4B. However, the ratio between the two becomes

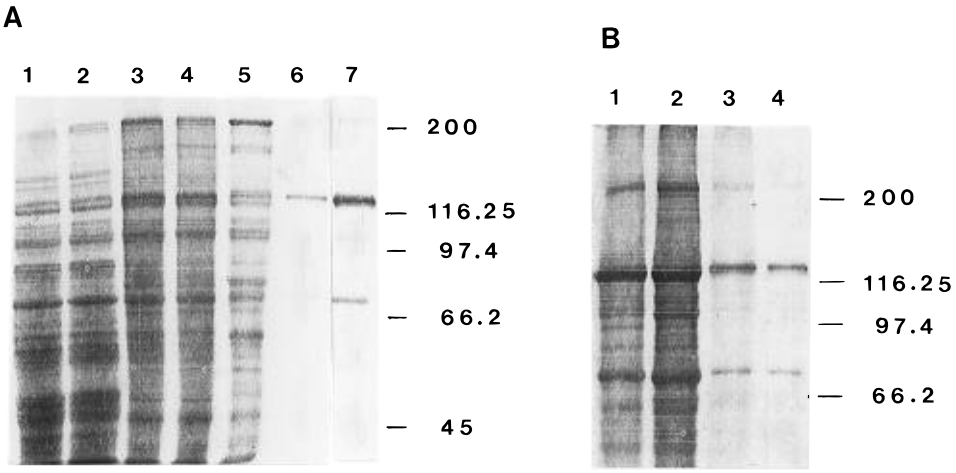

FIGURE 2: Purification of recombinant mNOS and C194S and C194H mutants. Panel A. Coomassie Blue-stained 7.5% SDS−PAGE gel: lane 1, 100 000*g* supernatant (80 μg); lane 2, supernatant after streptomycine sulfate treatment (75 μg); lane 3, ammonium sulfate pellet after G-25 desalting (57 μg); lane 4, affinity column flow-through (55 μg); lane 5, affinity column wash (15 μg), lane 6, = affinity column elution ($\approx$10 μg); lane 7, concentrated NOS after Sephacryl S300 gel-filtration(50 μg). Panel B. C194H and C194S mutated mNOS purification: lanes 1 and 2, 100 000*g* supernatant of C194H and C194S, respectively ($\approx$100 μg); lanes 3 and 4, C194H and C194S NOS after affinity column elution and gel-filtration on Sephacryl S300, respectively ($\approx$50 μg).

Table 2: Parameters Affecting the Catalytic Activity of mNOS

citrulline assay modifications	% of activity
standard	100 ± 25[a]
− NADPH	<1
− $CaCl_2$	70 ± 20
− calmodulin	70 ± 20
− FAD − FMN	55 ± 10
+ *S*-methylthiocitrulline (500 μM)[b]	<1
+ NNA (100 μM)	12 ± 5[c]

[a] Citrulline formation was monitored using the Dowex assay (experimental part) with 100 μM radiolabeled L-arginine. The values reported are the mean of experiments performed on three different preparations of enzyme. Each assay was run for 8 min, and aliquots were taken every 3 min for analysis. Under every condition described, linearity was observed for at least 10 min. 100% activity corresponds to 130 ± 50 nmol of citrulline min^{-1} (mg of protein)$^{-1}$. [b] Prepared according to Narayanan and Griffith (1994). [c] Inhibition values reported for NNA were the results of experiments performed with 10 μM L-arginine.

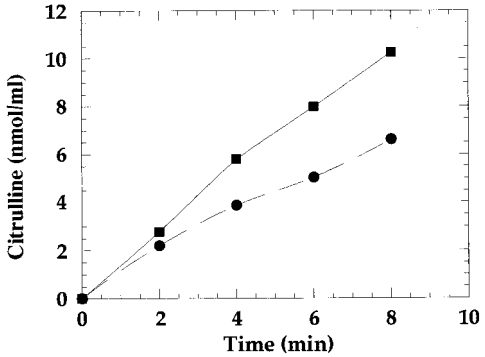

FIGURE 3: Production of citrulline by purified recombinant mNOS monitored as a function of time in presence (5 μM) (■) or absence of BH4 (●). Concentration of L-arginine was 100 μM, and the assay was performed as described in Experimental Procedures (5−10 μg of protein per assay).

steadily greater as a function of time. This result is in agreement with several literature data which are in favor of a protection of NOS by tetrahydrobiopterin (Giovanelli et al., 1991; Cho et al., 1992). Kinetic characterization of recombinant mNOS reveals a K_m for arginine of 7 ± 3 μM, which is not far from the values found for purified mNOS

Table 3: Compared Characteristics of Recombinant mNOS and Its C194S and C194H Mutants[a]

	mNOS	C194H	C194S
K_m for L-arginine (μM)	7 ± 3	ND	ND
V_m [nmol of citrulline min^{-1} mg of protein)$^{-1}$]	130 ± 50	<2	<2
K_m for cytochrome *C* (μM)	34 ± 8	43	45
V_m [μmol of reduced cytochrome *C* min^{-1} (mg of protein)$^{-1}$]	25 ± 5	20	18
heme visible spectrum (λ_{max})	393	none	none
difference visible spectrum of the Fe(II)−CO complex (λ_{max})	446	none	none
recognition with anti-i-NOS	yes	yes	yes
mg of purified protein per liter of culture	0.05−0.1	$\approx$0.3	$\approx$0.3

[a] Determinations for mNOS are the average of three to five independent preparations, whereas determinations for the mutated enzymes were performed on two preparations. Up to 0.5 mg of the mutated enzymes was used in spectroscopy measurements to make sure that no Fe(II)−CO spectrum could be recorded.

in the literature (Stuehr & Griffith, 1992; Griffith & Stuehr, 1995). Its V_m of 130 ± 50 nmol of citrulline min^{-1} (mg of protein)$^{-1}$ (Table 3) is eight times lower than the best reported specific activities of mNOS isolated from macrophage cell lines (Stuehr & Griffith, 1992; Griffith & Stuehr, 1995). This lower specific activity of recombinant mNOS is only partly due to the noncomplete purification of the enzyme (see Figure 2A). It could also come either from a monomer−dimer equilibrium not completely shifted toward the active dimeric form (Baek et al., 1993), or from a yeast calmodulin−mNOS complex less active than the mouse calmodulin−mNOS complex present in mNOS purified from mouse macrophages. Further experiments would be necessary to explain this lower specific activity of yeast-expressed mNOS. Nevertheless, it is noteworthy that it is eight times higher than the best activities published so far for a recombinant mNOS (Nakane et al., 1995). K_m and V_m values reported in Table 3 have been determined with the citrulline Dowex assay, but values obtained with the hemoglobin assay are in the same range [V_m: 100 nmol min^{-1} (mg of protein)$^{-1}$]. Recombinant purified mNOS is also active for the reduction of cytochrome *C* by NADPH with K_m and V_m values [34 ± 8 μM and 25 ± 5 μmol of cytochrome *C*

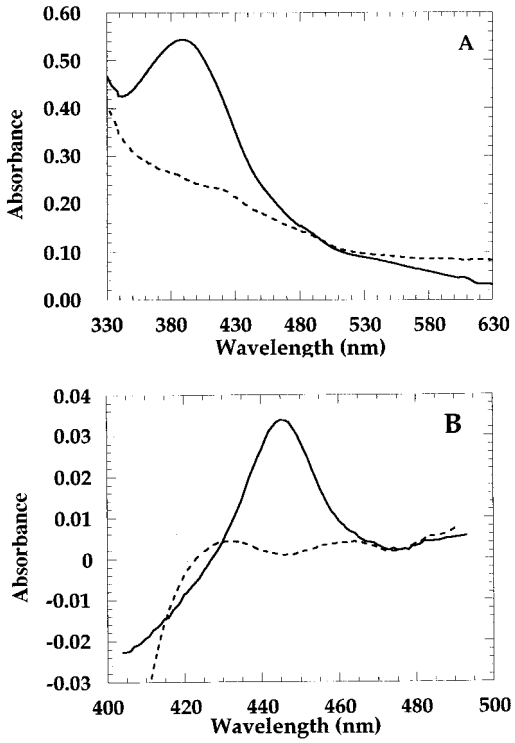

FIGURE 4: UV−visible spectroscopy of the purified recombinant wild type or C194S mutant mNOS. Panel A. Absolute spectrum of the purified enzyme (≈500 μg) recorded between 330 and 630nm. Panel B. Difference spectrum of the purified enzyme (≈20 μg) in the presence of dithionite and CO. Solid line, wild type NOS; dashed line, C194S mutant NOS (for the mutant NOS up to 500 μg was used to make sure no absorbance was present between 390 and 410 nm). Results obtained for the C194H mutant were identical to those shown for C194S.

reduced min^{-1} (mg of protein)$^{-1}$; Table 3] which are very similar to those previously published for mNOS [40 μM and 23 μmol min^{-1} (mg of $protein^{-1}$); Baek et al., 1993].

Finally, the recombinant mNOS displays a visible difference spectrum characterized by a Soret peak at 393 nm and a difference visible spectrum for its Fe(II)−CO complex with a Soret peak at 446 nm (Figure 4), in agreement with the spectra described for the enzyme purified from macrophages (White & Marletta, 1992; Stuehr & Ikeda-Saito, 1992).

Preparation and Characterization of mNOS Site-Directed Mutants. Comparison of the sequences of NOSs and cytochromes P450 has led several laboratories to suggest that a conserved nonapeptide sequence in NOSs could be the equivalent of the decapeptide in P450s which contains the proximal cysteine and appears to be a major element in heme binding (McMillan et al., 1992; Richards & Marletta, 1994; Renaud et al., 1993). On the basis of this comparison, the proximal cysteine in NOSs should be cysteine 415 in rat brain NOS, cysteine 184 in human endothelial NOS, and cysteine 194 in murine macrophage NOS. The corresponding site-directed mutants of rat brain NOS expressed in baculovirus-infected insect cells (C415H; Richards & Marletta, 1994) and of endothelial NOS expressed in COS cells (C184A; Chen et al., 1994) were found to be unable to oxidize L-arginine to citrulline. Moreover, these mutant proteins did not display any significant absorbance in the 450-nm region when reduced in the presence of CO, a characteristic of heme-thiolate proteins. Experiments of this nature have not been performed on mNOS due to the unavailability of a heterologous expression system that gives adequate quantities of active protein for proper characterization. In order to address whether cysteine 194 of mNOS is the proximal heme ligand, as well as to highlight our yeast-based expression system, site-directed mutants C194H and C194S were constructed.

Two pMacNOS60 plasmids coding for these mutants were constructed using a two-round PCR process. Each mutated plasmid was then used to transform the W(R)*fur1* yeast strain and processed as described above to generate the pMacNOSC194H/W(R)*fur1* and pMacNOSC194S/W(R)*fur1* yeast strains. As previously mentioned, the strain expressing wild type NOS was not able to grow on an inducible galactose-containing minimal medium but was fully able to grow on a non-inducible glucose-containing medium. In contrast, strains expressing the C194H or the C194S mutated mNOS were fully able to grow on both media with equal efficiency.

The W(R)*fur1* strains expressing the mutant NOSs were grown on a rich medium containing ethanol, and induction was started by adding galactose to the saturated culture as described above for the wild type strain. Purification of the mutant enzymes was carried out as described for the wild type mNOS except that detection of the protein eluted from the S300 column was at 280 nm instead of 400 nm. In fact, the mutant proteins were yellow whereas wild type NOS was green. Interestingly, expression levels of the mutated proteins were higher than that of the wild type protein (Table 3). As shown on Figure 2, there was no difference between the wild type and mutant recombinant NOSs on SDS−PAGE, and an antibody raised against an inducible NOS was equally capable of recognizing the mutant forms of the enzyme. Moreover, both mutants were able to reduce cytochrome *C* with V_m and K_m values very similar to those found for the wild type enzyme (Table 3). As expected, the mutations in the oxygenase domain of mNOS did not affect the reductase activity of its flavoprotein domain. Alternatively, none of the mutated enzymes was able to catalyze the oxidation of L-arginine to citrulline. The limit of detection of the assay, performed as described above, was 1.5% that of the wild type mNOS activity (Table 3). Finally, the visible spectrum of the two mutants did not show any absorbance around 400 nm, which would have been characteristic of the presence of heme (Figure 4). Moreover, their difference visible spectra in the presence of dithionite and CO failed to reveal any absorbance around 450 nm (Figure 4).

These data show that mNOS mutants in which cysteine 194 was replaced with either histidine or serine are well expressed in W(R)*fur1* strain (even better than wild type mNOS) and are as catalytically active as the wild type enzyme for the reduction of cytochrome *C*. However, they appear to be unable to bind heme and, consequently, they are completely inactive toward the oxidation of L-arginine. These data provide a first experimental evidence in favor of cysteine 194 as the heme proximal ligand of mNOS. Altogether these results and those in favor of Cys 415 and Cys 184 as heme proximal ligand of rat brain NOS and human endothelial NOS, respectively (Richards & Marletta, 1994; Chen et al., 1994), strongly support the proposition of a highly conserved sequence of NOS as the heme binding segment (McMillan et al., 1992; Richards & Marletta, 1994; Mansuy & Renaud, 1995).

CONCLUSION

The expression of murine macrophage NOS in *S. cerevisiae*, using classical technology, was found to be cytotoxic for the host. However, by use of a yeast strain engineered to allow expression of the heterologous protein at a high cell density and a three-step procedure that allows yeast growth and mNOS expression to be uncoupled, it was possible to partly avoid cytotoxic effects and achieve mNOS expression at a reasonable level. The cytotoxic effects observed during mNOS expression are likely to be mediated by ·NO or NO-derived products since mutant NOSs that were unable to produce ·NO were not toxic to the host. Furthermore, purifications carried out on the mutant strains indicate that the mutant proteins were expressed at higher levels than the wild type protein (Table 3). In that regard, we have recently achieved the expression of rat neuronal NOS using identical technology. In this case, no toxicity was observed and the expression level of nNOS was at least 20 times higher than that of mNOS [Sari et al., unpublished data; see also results of Black & Ortiz de Montellano (1995) on nNOS expression in another yeast strain]. Interestingly, no NOS activity was detected in the 100 000*g* supernatant of yeast expressing rat nNOS unless calmodulin was added to the extract, whereas mNOS was functional in yeast cytosol without addition of external calmodulin. It is thus likely that yeast calmodulin is capable of activating mNOS whereas it is not able to correctly activate nNOS, presumably because of dramatic differences in its relative affinity for the two NOSs. The ·NO-related cytotoxicity could play a central role in the much lower level of expression of mNOS relative to nNOS. Nevertheless, the expression level of mNOS is sufficient to allow the protein to be routinely purified, be characterized by visible spectroscopy, and be obtained under a catalytically active form. This expression system should be useful for further studies on mNOS as it has been already used to show that the proximal ligand of the mNOS heme iron is consistent with cysteine 194.

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