Neuronal nitric-oxide (NO) synthase contains FAD, FMN, heme, and tetrahydrobiopterin as prosthetic groups and represents a multifunctional oxidoreductase catalyzing oxidation of L-arginine to L-citrulline and NO, reduction of molecular oxygen to superoxide, and electron transfer to cytochromes. To investigate how binding of the prosthetic heme moiety is related to enzyme activities, cofactor, and L-arginine binding, as well as to secondary and quaternary protein structure, we have purified and characterized heme-deficient neuronal NO synthase. The heme-deficient enzyme, which had preserved its cytochrome c reductase activity, contained FAD and FMN, but virtually no tetrahydrobiopterin, and exhibited only marginal NO synthase activity. By means of gel filtration and static light scattering, we demonstrate that the heme-deficient enzyme is a monomer and provide evidence that heme is the sole prosthetic group controlling the quaternary structure of neuronal NO synthase. CD spectroscopy showed that most of the structural elements found in the dimeric holoenzyme were conserved in heme-deficient monomeric NO synthase. However, in spite of being properly folded, the heme-deficient enzyme did bind neither tetrahydrobiopterin nor the substrate analog N⁶-nitro-L-arginine. Our results demonstrate that the prosthetic heme group of neuronal NO synthase is requisite for dimerization of enzyme subunits and for the binding of amino acid substrate and tetrahydrobiopterin.

Nitric oxide, an important effector and signaling molecule in the nervous, immune, and cardiovascular systems (1–4), is enzymatically generated from L-arginine and molecular oxygen in the nervous, immune, and cardiovascular systems (1–4), is enzymatically generated from L-arginine and molecular oxygen by different nitric-oxide synthases (NOS) (5). The isozyme purified from brain (5–7) termed neuronal NOS (nNOS), was identified as a 320-kDa homodimer, containing close to stoichiometric amounts of heme, FAD, and FMN as well as variable amounts of tetrahydrobiopterin (H₄biopterin) (8–14). nNOS exhibits sequence similarities to cytochrome P450 reductase (15) and to the heme-binding sequence in P450 proteins (16), suggesting that the enzyme represents a fusion protein of a cytochrome P450-like protein and a P450 reductase (17). In support of this hypothesis, nNOS was shown to catalyze the reduction of cytochrome c (18) and to possess a cysteine-ligated heme-iron (19, 20) with spectral properties characteristic of a P450 protein (9, 12, 21). Further studies have confirmed the bidomain structure of nNOS (20, 22, 23), with the reductase domain shuttling NADPH-derived electrons in a calmodulin-triggered fashion from the flavins to the heme moiety (24, 25), which is located in close proximity to the amino acid substrate and pteridine binding sites (26) and catalyzes a two-step oxygenation of L-arginine to L-citrulline and NO with N⁶-hydroxy-L-arginine as intermediate (27, 28).

Recent work has focused on the allosteric regulation of NOS by L-arginine, H₄biopterin, and heme. The cytokine-inducible NOS dimer from macrophages was shown to form catalytically inactive monomers in the absence of H₄biopterin or L-arginine and, notably, to lose its prohormetic heme group in the absence of these ligands. Reassociation of the monomers to catalytically active dimers required the coincident presence of heme, L-arginine, and pteridine, pointing to a role of these compounds in the post-translational processing of inducible NOS (29). However, in contrast to the inducible isozyme, native nNOS maintained its catalytically active, dimeric conformation even in the absence of pteridine and L-arginine (14). Thus, it is currently unclear how binding of heme to nNOS is related to subunit assembly, catalytic activity, and pteridine binding. To address this issue, we have purified heme-deficient nNOS (nNOS(h⁻)) from a baculovirus overexpression system and analyzed the virtually heme-free protein for catalytic activity, cofactor content, amino acid substrate, and H₄biopterin binding and determined the secondary and quaternary structural features of the enzyme.
EXPERIMENTAL PROCEDURES

Materials—L-[2,3,4,5-3H]Arginine hydrochloride (57 Ci/mmol) and N^4-arginino-[2,3,4,5-3H]arginine hydrochloride (56 Ci/mmol) were purchased from MedPro (Amersham), Vienna, Austria. Labeled l-arginine was further purified by cation exchange HPLC with 50 mM sodium acetate (pH 5.5) as eluent to reduce blank levels in the citrulline assay. 3–6 (R)-5,6,7,8-[3H]Tetrahydrobiopterin (14 Ci/mmol) was synthesized enzymatically from [8,5,6,7,8-3H]GTP as described previously (30). Pteridines were obtained from Dr. B. Schirlich Laboratories, Ann Arbor, Switzerlan. All other chemicals were from Sigma-Aldrich Chemie, Vienna, Austria or from sources described previously (8, 31, 32).

Preparation of nNOS—Large scale purification of heme-saturated nNOS (nNOS(h^-)) had a concentration of 0.9 eq of heme per monomer from ~4.5 x 10^9 S9 cells infected in the presence of heme chloride (4 mg/liter) with rat brain nNOS-recombinant baculovirus (32) was performed by sequential affinity chromatography on 2.5-ADP-Sepharose and calmodulin-Sepharose as described in detail recently (33). Partially heme-deficient nNOS (~0.3 eq of heme per monomer) was obtained from S9 cells, which had been infected in the absence of heme chloride. For the isolation of NOS(h^-), the partially heme-deficient protein was concentrated to ~0.10 mM by means of Vivapore concentrators (Vivascience Ltd., Stonehouse, UK), and 0.20-mM aliquots were subjected to gel filtration chromatography as described below. Fractions (0.3 ml) containing ~0.40 nmol of nNOS (<0.1 eq of heme per monomer) were pooled and stored at ~70°C. Purified nNOS(h^-) had a concentration of ~4.5 μM and contained ~0.04 eq of heme per monomer.

Gel Filtration Chromatography—Aliquots of 0.10–0.20 ml containing 0.5–20 nmol of nNOS were injected into an HPLC system (Lichrocart 100–5, 10.5 cm x 0.46 cm; LichroCart 100–10, 10.5 cm x 0.4 cm) equipped with a low pressure gradient controller (L-4250, Merck). Fractions of 0.30 ml were collected for protein determinations and final volumes of 0.20 ml of 50 mM triethanolamine/HCl buffer, pH 7.0. Reaction mixtures were incubated in the absence or presence of H4biopterin (0.1 mM) and 5–100 μM L-arginine as described previously (8, 18). Kinetic parameters, substrate concentrations were 1–50 μM and 5–100 μM (cytochrome c, respectively. Rates of NADPH oxidation and nNOS activity were calculated using extinction coefficients of 0.3 and 21 mM-1 x cm-1, respectively. For the determination of enzyme kinetic parameters, substrate concentrations were 1–50 mM (L-arginine) and 5–100 μM (cytochrome c), respectively. Vmax and Km values were obtained from weighted Lineweaver-Burk plots (37). Turnover numbers (kcat) were calculated from the respective Vmax values and represent the maximum number of converted –l-arginine molecules per min and number of active sites, i.e., the number of heme-containing NOS molecules.

Radioligand Binding Studies—Binding experiments were performed as described previously (38, 39). Briefly, nNOS (18–40 pmol) was incubated for 10 min at 37°C with 12 μM l-[4-3H]NNA (70 nCi) or [14C]biopterin (77 nCi) and increasing concentrations of the respective unlabeled ligand (10 nm-10 μM) in 0.1 ml of a 50 mM triethanolamine/HCl buffer, pH 7.0. Reactions were stopped by polyethylene glycol precipitation followed by vacuum filtration. The amount of the bound radioligand retained on the filters was determined by liquid scintillation counting. Data were corrected for nonspecific binding determined in the presence of 1 μM unlabeled ligand. Kd and Bmax values were calculated using the nonlinear least squares curve fitting program (40). Given the low overall recovery (~25%) of this binding assay (36, 38, 39), the calculated Bmax values are only semi-quantitative estimates and do not allow the determination of the number of pteridine or l-arginine binding sites.

Gel Electrophoresis—nNOS was subjected to low temperature SDS-PAGE essentially as described recently (14). Briefly, nNOS (~150 pmol) was incubated for 5 min at 37°C in 50 μl of 50 mM triethanolamine/HCl buffer (pH 7.0) in the absence or presence of H4biopterin (0.1 mM) and L-arginine (1 mM). Incubations were terminated by the addition of 50 μl of chilled Laemmli buffer (41), containing 0.125 Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (w/v) glycerol, and 0.02% (w/v) bromophenol blue. 2-Mercaptoethanol, which is included in the lysis buffer as described above, was omitted from the Laemmli buffer without having any effect on the migration pattern nNOS. Samples, containing ~30 pmol of nNOS, were subjected to SDS-PAGE for 60 min at constant current of 30 mA on discontinuous 6% SDS-gels (70 x 80 x 1 mm). Gels and buffers, which had been prepared according to Laemmli (41), were equilibrated at 4°C prior to electrophoresis, and the buffer tank was cooled with means of an ice bath during electrophoresis. Gels were then stained for protein with Coomassie Blue R-250 or for heme with 3,3'-diaminobenzidine dihydrochloride in methanol. Gels were developed for 60 min by adding H2O2 to a final concentration of 60 mM, washed for 30 min in H2O/ methanol/acetic acid = 8:1:1 (v/v/v), dried, and photographed. The amount of protein or heme were estimated by densitometric analysis using the vds 800 video system and H1D-software of Hirschmann Analysetechnik.

Reconstitution of nNOS—Reconstitution of L-[2,3,4,5-3H]arginine was successful, as indicated by a light scattering increase (43, 44). Accurate determinations of the specific refractive index increments (dn/dc) of the solute particles are required for molecular mass determinations by this method. Due to limitations in the amount of the available NOS (nNOS(h^-) protein, related to 15 μM H4biopterin (5 μM H2O2), the amount of nNOS(h^-) protein was limited. Therefore, the dn/dc values for related proteins are identical, and there is no appreciable angular dependence of the scattering intensity (I0 I0). Thus, the scattering intensity of different nNOS species measured at a fixed scattering angle depends exclusively on (molecular mass x protein concentration), allowing the determination of the molecular mass ratio of nNOS(h^-) and nNOS(h+). Light scattering measurements were performed on an arbitrary scale with equally concentrated solutions of nNOS(h^-) and nNOS(h+) (5.4 μM) in rectangular microwell vials (30 μl) at a scattering angle of 90 degrees at 8°C, using an Ar+-
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RESULTS

To investigate the role of heme in nNOS structure and function, we attempted to purify recombinant heme-deficient nNOS from a baculovirus overexpression system. From $4.5 \times 10^9$ cells, which had been infected with the rat brain NOS-recombinant baculovirus for 48 h in the absence of hemin chloride, we obtained $\approx 80$ mg of nNOS with a heme content of 0.29 ± 0.03 eq per monomer ($n = 3$). The amount of heme bound to the recombinant protein was not significantly reduced by preincubation (24–48 h) and infection (48 h) of Sf9 cells in the presence of 0.25 and 0.50 mM succinyl acetone, an inhibitor of heme biosynthesis, although cytosolic heme levels were reduced down to 10% of untreated control cells under these conditions (not shown). Partially heme-deficient nNOS was further analyzed for H$_4$bipterin, FAD, and FMN, revealing that substoichiometric amounts of these cofactors were incorporated into the protein (0.12 ± 0.01, 0.17 ± 0.01, and 0.11 ± 0.01 eq per monomer, respectively; $n = 3$). In the presence of saturating concentrations of exogenous H$_4$bipterin, FAD, and FMN, the partially heme-deficient enzyme exhibited only low specific activity (0.30 ± 0.03 mol of L-citrulline × min$^{-1} \times$mg$^{-1}$, $n = 3$) as compared with the holoenzyme (1.08 ± 0.14 mol of L-citrulline × min$^{-1} \times$mg$^{-1}$, $n = 3$).

To investigate whether partially heme-deficient nNOS can be reconstituted with flavins, we have reconstituted the protein with a 2-fold molar excess of FAD and FMN for 5 min at room temperature and subsequently removed free flavins by gel filtration chromatography. Under these conditions, the amount of protein-bound FAD and FMN was increased -6-fold to 0.98 ± 0.06 and 0.65 ± 0.04 eq per monomer, respectively ($n = 3$). Increasing the incubation time to 60 min did not further enhance FMN binding. To confirm that the flavins were incorporated into the nNOS protein in a catalytically active form, we have determined cytochrome c reductase activities of the reconstituted protein in the absence of exogenous flavins. Enzyme kinetic analysis revealed that reconstitution of nNOS with flavins had no effect on the affinity of cytochrome c for nNOS but was accompanied by a -5-fold increase of cytochrome c reductase activity as compared to controls ($K_{m, c} = 1.4 ± 3 \mu$M; $V_{max} = 2.6 ± 0.6$ versus $15 ± 2 \mu$mol of cytochrome c × mg$^{-1}$ × min$^{-1}$; $n = 3$). Maximal rates of L-citrulline formation as well as L-arginine- and H$_4$bipterin-independent NADPH oxidation were not affected (not shown), demonstrating that the amount of enzyme-bound heme was limiting for NOS activity of the protein.

Gel filtration chromatography of the partially heme-deficient enzyme preparations, which had been reconstituted with FAD and FMN, revealed that NO synthase activity eluted in a single and well defined peak centered at an elution volume of 11.8 ml (Fig. 1, upper panel, filled circles). nNOS eluting in this peak fraction contained 0.56 ± 0.06 eq of heme per monomer ($n = 3$) and had a specific activity of 0.88 ± 0.09 mol × min$^{-1} \times$mg$^{-1}$ (n = 3) when assayed in the presence of saturating concentrations of exogenous H$_4$bipterin and flavins. Cytochrome c reductase activity eluted in a broad peak with two maxima at 11.8 and 13.0 ml, and -40% of the total reductase activity were found in fractions which did not exhibit detectable citrulline formation (Fig. 1, upper panel, open circles), suggesting the separation of two protein species. As shown in the lower panel of Fig. 1, comparable elution profiles were found when the eluate was assayed for protein (filled circles), FAD (open circles), and FMN (filled squares), demonstrating that both protein species contained virtually stoichiometric amounts of FAD and -0.6 eq of FMN per monomer. Substoichiometric binding of FMN as well as a rightward shift of the FMN elution profile points to dissociation of the flavin during gel filtration. In contrast to FAD and FMN, which co-eluted with the reductase activity of nNOS, heme and H$_4$bipterin were strictly co-eluted with L-citrulline forming activity, demonstrating that heme-deficient nNOS was separated from the active heme-containing enzyme.

![Fig. 1. Gel filtration chromatography of partially heme-deficient nNOS.](http://www.jbc.org)
and 0.007 ± 0.001 eq per monomer, respectively; n = 3). The amount of flavins bound to nNOS(h−) was almost identical with that of the starting material used for the isolation of the heme-deficient enzyme (0.87 ± 0.14 versus 0.98 ± 0.06 and 0.61 ± 0.08 versus 0.65 ± 0.04 eq per monomer, respectively; n = 3), indicating that heme deficiency does not affect the affinity of nNOS for FAD and FMN.

Enzyme kinetic parameters for nNOS(h−) and nNOS(h−) were calculated from weighted Lineweaver-Burk plots obtained by determining rates of l-citrulline formation in the presence of saturating concentrations of H4biopterin and flavins and increasing concentrations of l-arginine (1–50 μM). Under these conditions, nNOS(h−) exhibited only very little NOS activity (Vmax = 26 ± 4 nmol l-citrulline min−1 × mg−1, n = 3) as compared with nNOS(h+) (Vmax = 1.2 ± 0.1 μmol of l-citrulline min−1 × mg−1, n = 3), whereas the affinity of both protein species for l-arginine was comparable (Km = 7.2 ± 0.6 μM (nNOS(h−)) versus 5.5 ± 0.4 μM (nNOS(h+)); n = 3), indicating that residual NOS(h−) present in preparations of the heme-deficient enzyme accounts for the observed NOS activity. In support of this, we found that turnover numbers (kcat), which had been corrected for the heme content of the protein preparations, did not significantly (p = 0.09, unpaired t test) differ (nNOS(h−): 0.030 ± 0.006 eq of heme per monomer, kcat = 139 ± 35 min−1 (n = 3); nNOS(h+): 0.93 ± 0.05 eq of heme per monomer, kcat = 206 ± 20 min−1 (n = 3)).

We have recently shown that nNOS purified from porcine brain is converted to an SDS-resistant dimer upon binding of H4biopterin and l-arginine (14). Heme saturation of the porcine enzyme (13) precluded, however, investigation of the contribution of the prosth tic heme group to this tight interaction of nNOS subunits. To address this issue, we have subjected nNOS(h−) and nNOS(h−), which had been preincubated with 2% SDS in the absence and presence of H4biopterin (0.1 mW) and l-arginine (1 mW), to low temperature SDS-PAGE. In the absence of the added ligands (Fig. 2, upper panel, lane A), the ratio of SDS-resistant nNOS(h+) dimers (∼300 kDa) to monomers (∼150 kDa) was ∼15:85. Preincubation of nNOS(h+) with exogenous H4biopterin (0.1 mW) and l-arginine (1 mW) increased the relative amount of dimeric nNOS to ∼50%. With nNOS(h−), we did not detect any SDS-resistant protein dimers both in the absence (lane C, upper panel) and presence (lane D, upper panel) of saturating amino acid substrate and cofactor concentrations. Heme staining of the gels by means of the dimethoxybenzidine/H2O2 method (Fig. 2, lower panel) shows that the ratio of heme-containing nNOS(h+) dimers to monomers was ∼90:10 in the absence of H4biopterin and l-arginine (lane A, lower panel) and, thus, markedly higher than that determined by means of protein staining (see lane A, upper panel). Preincubation in the presence of exogenous pteridine and l-arginine led to virtually complete dimerization of the heme-containing protein (lane B, lower panel), demonstrating that ∼50% of nNOS(h−) had lost their prosthetic heme group during electrophoresis. As expected, nNOS(h−) monomers did not stain for heme (lanes C and D, Fig. 2, lower panel).

To investigate the involvement of the prosthetic heme group in the dimerization of nNOS under native conditions, we have analyzed nNOS(h+) and nNOS(h−) by means of gel filtration chromatography and static light scattering. In the course of gel permeation chromatography on Superose 6, heme-saturated (0.90 eq of heme per monomer) and heme-deficient (0.05 eq of heme per monomer) nNOS eluted at 11.6 and 13.1 ml, respectively (Fig. 3A). From these data, we calculated Stokes radii of 6.3 ± 0.3 and 8.1 ± 0.1 nm (n = 3) for nNOS(h+) and nNOS(h−), respectively (Fig. 3B), showing that the hydrodynamic volume of nNOS(h+) (1.05 ± 0.05 × 10−24 m3) is 2.1 ± 0.1-fold smaller than that of the holoenzyme (2.23 ± 0.03 × 10−24 m3). Together with the observation that the native, heme-saturated holoenzyme forms a 320-kDa homodimer (11, 12), our results suggest that nNOS(h−) is a 160-kDa monomer. Alternatively, the smaller Stokes radius of nNOS(h−) may result from a more compact, globular conformation compared to the elongated holoenzyme, which was shown to exhibit an axial ratio of ∼20:1 (14). This was clarified by means of static light scattering, a technique which allows us to determine the molecular mass ratio of related macromolecules in solution.
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FIG. 4. Reconstitution of nNOS(h−) with heme. nNOS(h−) (4 μM) was incubated in the absence and presence of 8 μM hemin chloride for 30 min at ambient temperature in a 50 mM triethanolamine/HCl buffer, pH 7.0, containing 12 mM mercaptoethanol. Aliquots (100 μl, 0.4 nmol of nNOS) were injected onto a Superose 6 gel filtration column and eluted as described under "Experimental Procedures." Protein in the eluate was monitored at 280 nm. The chromatogram shown is representative of three. Inset, column fractions of 0.30 ml were assayed for heme by HPLC as described under "Experimental Procedures." Data are the total amount of heme co-eluting with nNOS and represent means ± S.E. of three separate experiments performed with two protein preparations.

Specific mean scattering intensities of equally concentrated solutions (5.4 ± 0.4 μM) of nNOS(h+) and nNOS(h−) were 16 ± 0.2 × 10⁶ and 8.3 ± 1.0 × 10⁶ counts s⁻¹ M⁻¹ (n = 2, 20 scans each), respectively (Fig. 3C), demonstrating that the molecular masses of nNOS(h−) and nNOS(h+) differed by a factor of 1.9 ± 0.3.

To determine whether reconstitution of nNOS(h−) monomers with heme results in the reassociation of enzyme subunits into a homodimer, we have incubated heme-deficient monomers (4 μM) with a 2-fold molar excess of heme chloride for 30 min at ambient temperature prior to removal of unbound heme by gel filtration chromatography (Fig. 4). As estimated from the peak areas, the relative amount of dimeric nNOS was increased ~10-fold from ~5% to ~55% upon incubation with heme. The inset to Fig. 4 shows that dimerization of nNOS subunits was accompanied by an ~10-fold increase in protein-bound heme from 20 ± 9 pmol to 195 ± 35 pmol (n = 3) which corresponded to a heme content of 0.5 eq of per monomer. Increasing the incubation time to 60 min, varying the protein: heme ratio from 1:1 to 1:5, or co-incubation with L-arginine and H₂bioppterin (8 μM each) gave essentially the same results (data not shown). Reconstitution with heme in the presence or absence of H₂bioppterin/L-arginine did not restore enzyme activity as determined by L-arginine-independent NADPH oxidation and formation of L-citrulline from L-arginine (not shown).

Interestingly, the amount of H₂bioppterin bound to nNOS(h−) (0.007 ± 0.001 eq per monomer) was markedly reduced as compared with the heme-saturated enzyme (0.45 ± 0.03 eq per monomer). To find out whether enzyme-bound heme is requisite for pteridine binding, we have performed binding studies with nNOS(h+) and nNOS(h−) using [³H]H₂bioppterin as radioligand (Fig. 5A). In accordance with previous studies performed with nNOS from porcine brain (36, 38), nNOS(h−) bound [³H]H₂bioppterin with Kᵦ and Bₘ values of 0.26 ± 0.01 μM and 129 ± 3 pmol per nmol of monomer (n = 3), respectively (panel A, solid symbols), whereas only marginal amounts of the pteridine bound to the heme-deficient enzyme (panel A, open symbols; Bₘ < 10 pmol per nmol of monomer, n = 3). Since the amino acid substrate site of nNOS appears to interact with the heme domain of the enzyme (48, 49), we further investigated the role of the prosthetic heme group in binding of high affinity amino acid substrate analog L-[³H]NNA (39). As shown in panel B of Fig. 5, nNOS(h−) bound L-[³H]NNA with an efficacy closely similar to that observed with [³H]H₂bioppterin (closed symbols; Bₘ = 133 ± 2 pmol per nmol of monomer, Kᵦ = 0.17 ± 0.02 μM; n = 3), whereas binding to nNOS(h+) was negligible (open symbols; Bₘ < 10 pmol per nmol of monomer, n = 3). It should be pointed out that the binding assay is not quantitative and does not allow, therefore, to determine the absolute amount of bound H₂bioppterin (see "Experimental Procedures").

The apparent loss of amino acid substrate and pteridine binding sites in nNOS(h−) suggests that heme deficiency may be accompanied by unfolding of the protein. To address this issue, we have analyzed nNOS(h+) and nNOS(h−) by CD spectroscopy. As shown in Fig. 6, nNOS(h+) displayed a well-defined far-UV CD spectrum with minima at 208 and 220 nm, a maximum at 192 nm, and baseline crossovers at 200 nm and ~180 nm (solid line). Similar spectra were obtained for the heme-deficient enzyme (dashed line), and secondary structure analysis revealed that both nNOS species were virtually identical with regard to their content of parallel β-sheet, turns and other structures (Table I). However, nNOS(h−) and nNOS(h+) differed slightly in their content of α-helical structures (0.27 versus 0.34) and antiparallel β-sheet (0.15 versus 0.11), pointing to subtle secondary structure changes occurring upon heme binding.

DISCUSSION

It was the objective of the present study to isolate and characterize heme-free nNOS in order to find out how binding of the prosthetic heme group affects the catalytic and structural features of the enzyme. Infusion of SF9 cells with rat nNOS-recombinant baculovirus in the absence of added hemin yielded a partially heme-deficient nNOS which was purified and subjected to gel filtration chromatography for separation of the heme-free protein from the holoenzyme. Consistent with the essential role of heme in NOS catalysis, nNOS(h−) exhibited only marginal NOS activity, which was apparently due to contamination with holoenzyme (~7% of total protein). Heme deficiency neither affected the kinetic parameters for cytochrome
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Results from gel filtration chromatography and static light scattering revealed that the hydrodynamic volume and accordingly the molecular mass of nNOS(h−) was about half of the respective values calculated for nNOS(h+). Based on the identification of latter species as 320-kDa homodimer (7, 14), these data demonstrate that nNOS(h−) is monomeric. Reconstitution of nNOS(h−) with hemin resulted in pronounced incorporation of heme and consequent enzyme dimerization. However, the reconstituted heme-containing dimers did not exhibit detectable NOS activity, indicating that co-translational heme binding is essential for expression of catalytically active NOS.

In striking contrast with inducible NOS dimerization, which was reported to require the coincident presence of heme, binding is essential for expression of catalytically active NOS. Based on the variable selection method, using a basis set of 33 reference proteins (46). The secondary structure of nNOS(h+) and nNOS(h−) were computed from a final set of 29 combinations constructed from 29 protein spectra each and a set of 12 combinations including 24 spectra, respectively. Data represent the average structural content calculated from these selected combinations, in which the total of secondary structures was 1.00 and which gave a root-mean-square error of less than 0.20.

| Secondary structure | Average structural content nNOS(h+) | Average structural content nNOS(h−) |
|---------------------|-----------------------------------|-----------------------------------|
| α-Helix             | 0.27                              | 0.34                              |
| Antiparallel β-sheet| 0.15                              | 0.11                              |
| Parallel β-sheet    | 0.05                              | 0.05                              |
| Turns               | 0.19                              | 0.19                              |
| Others              | 0.33                              | 0.32                              |

nNOS(h+) dimers under comparable experimental conditions, i.e. upon preincubation with saturating concentrations of both H₄biopterin and L-arginine. As revealed by the heme-ion staining experiments, this incomplete dimerization was apparently due to loss of heme during SDS-PAGE of nNOS(h+). Thus, the enzyme obtained from porcine brain seems to bind the prosthetic heme group more tightly than the recombinant rat brain NOS. We are currently investigating whether this reflects species differences in the heme environment of NOS or results from a specific feature of the baculovirus overexpression system.

There is experimental evidence that the prosthetic heme group is part of the amino acid substrate site and interacts with the pteridine binding domain. Thus, L-arginine and H₄biopterin induce perturbations of the heme spectrum (20, 23, 48, 51), sequences located near the axial cysteine ligand of the heme are apparently involved in L-arginine and pteridine binding (26), and the heme site inhibitor 7-nitroindazole was found to antagonize L-arginine and H₄biopterin binding to NOS (36). In the present study, we demonstrate that nNOS(h+) binds H₄biopterin and the amino acid substrate analog L-NNA with high affinity, whereas binding of these ligands to the heme-deficient protein was negligible. The minor alterations in the CD spectrum point to an effect of heme deficiency on the secondary structure of the protein. Based on the CD spectra, it cannot be ruled out that the lack of binding activity of nNOS(h−) is due to a loss of secondary structure, but this appears to be unlikely because most of the structural elements found in dimeric nNOS(h+) were conserved in the heme-deficient monomer. As compared with the holoenzyme, nNOS(h−) contained slightly more α-helical structures accompanied by some loss of antiparallel β-strands, indicating that heme binding and concomitant subunit dimerization involve a subtle rearrangement of secondary structure necessary for formation of appropriately folded amino acid substrate and cofactor binding sites.

In conclusion, our data support a model for dimeric nNOS assembly as shown in Fig. 7. Heme-free NOS is monomeric, contains the flavins FAD and FMN, and exhibits only cytochrome c reductase activity. Depending on the intracellular availability of free heme, nNOS is expressed as a loosely associated homodimeric hemoprotein, which readily dissociates in the presence of SDS. In this conformation, nNOS acts as

Fig. 6. CD spectra of nNOS(h−) and nNOS(h+). CD spectra of nNOS(h−) (solid line) and nNOS(h+) (dashed line) were recorded at 20 °C with 12 μM protein in 50 mM sodium phosphate buffer, pH 7.4, as described under "Experimental Procedures." The spectra shown are representative of two.

Fig. 7. Role of heme in nNOS structure and function. nNOS(h−) represents a monomeric flavoprotein, catalyzing the reduction of cytochrome c and cytochrome P450 (18). The heme-deficient protein does not exhibit binding sites for L-arginine and H₄biopterin. Upon heme binding, the H₄biopterin-deficient enzyme forms a loosely associated homodimer, which functions as an NADPH oxidase, catalyzing the formation of superoxide and H₂O₂ (52, 53). Finally, this protein species is converted by H₄biopterin (H₄B) to a tight dimer exhibiting full NOS activity.
NADPH oxidase and catalyzes the formation of superoxide and H₂O₂. (52, 53). Binding of H₂biopterin to nNOS(++) further modifies the enzyme structure in that the subunits adopt a much tighter conformation resulting in the formation of super-
stable SDS-resistant dimers (14). This pteridine-induced con-
formational change, which may occur either co- or post-trans-
lational, is requisite for the coupling of reductive oxygen activation to L-arginine oxidation and, thus, for the conversion of the enzyme from an NADPH oxidase to a fully active NOS.

Accordingly, the role of the prosthetic heme group is not con-
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Characterization of Heme-deficient Neuronal Nitric-oxide Synthase Reveals a Role for Heme in Subunit Dimerization and Binding of the Amino Acid Substrate and Tetrahydrobioppterin

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