HEME INSERTION, ASSEMBLY, AND ACTIVATION OF APO-NEURONAL NITRIC OXIDE SYNTHASE \textit{IN VITRO}

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SUMMARY

It has been established that in the case of inducible NO synthase (NOS), a functionally active homodimer is assembled from the heme-deficient monomeric apo-NOS \textit{in vitro} by the addition of heme; whereas, the heme-deficient neuronal isoform (apo-nNOS) is at best only partially activated. In the current study we have discovered that reactive oxygen species, which can be removed by the addition of superoxide dismutase and catalase, destroy the heme and limit the activation of apo-nNOS \textit{in vitro}. With the use of these improved conditions, we show for the first time that heme insertion is a rapid process that results in formation of a heme-bound monomeric nNOS that is able to form the ferrous-CO P-450 complex, but is unable to synthesize NO. A slow process requiring over 90 min is required for dimerization and activation of this P450 intermediate to give an enzyme with a specific activity of 1100 nmol of NO formed/min/mg protein, similar to that of the native enzyme. Interestingly, the dimer is not SDS-resistant and is not the same dimer that forms \textit{in vivo}. These studies indicate at least two intermediates in the assembly of nNOS and advance our understanding of the regulation of nNOS.
INTRODUCTION

NO, which is synthesized by nitric oxide synthases (NOS) from L-arginine, O₂, and NADPH, is a key signaling agent in a variety of physiological processes (1-4). The enzyme is active as a homodimer with each monomer binding one equivalent of heme, FAD, FMN, BH₄, and Ca²⁺/calmodulin (5). The assembly or dimerization of inactive NOS monomers has been the topic of great interest since it may be an important regulatory step (6-19) and may afford a way to develop isoform selective inhibitors (20). Stuehr and coworkers (7) were first to show with the use of inducible NOS (iNOS) that the monomeric heme-deficient NOS could be functionally reconstituted to the dimeric form in vitro in the presence of L-arginine, BH₄, and stoichiometric amounts of heme. The activation of iNOS was a slow process requiring 90-120 minutes to reach the specific activity of fully functional iNOS. On the other hand, the assembly of heme-deficient monomeric neuronal NOS (apo-nNOS) by heme gave an inactive dimer (19). More recently, apo-nNOS was partially activated to roughly one-third of fully functional nNOS after 4 h of reconstitution with heme in degassed buffers (14). We wondered why there was at best only a partial activation of nNOS. As stated in a recent review (5), in light of the findings on iNOS, studies on the reactivation of nNOS are awaited with great interest. In the current study, we have found that reactive oxygen intermediates limit heme-mediated activation of apo-nNOS. When the formation of reactive oxygen intermediates were minimized we activated the enzyme with heme to the specific activity of the native enzyme. These improved reconstitution conditions allowed a more detailed study of the assembly of functional nNOS. Moreover, we have discovered that a heme-containing monomeric P450 is an early intermediate leading to the assembly of the active homodimer. Interestingly, the dimeric nNOS formed in vitro is not SDS-resistant and is not identical to the dimeric nNOS found in vivo. These findings shed new light on the mechanisms of assembly of nNOS in vitro that are likely to be important in the regulation of NO biosynthesis in vivo.
EXPERIMENTAL PROCEDURES

Materials

The cDNA for rat neuronal NOS was kindly provided by Dr. Solomon Snyder (Johns Hopkins Medical School, Baltimore, MD). (6R)-5,6,7,8-Tetrahydro-L-biopterin (BH$_4$) was purchased from Dr. Schirck’s Laboratory (Jona, Switzerland). Heme, myoglobin (horse heart), and NADPH were purchased from Sigma (St. Louis, MO). $^{14}$C-labeled heme (132 mCi/mole) was purchased from Leeds Radioporphyrins (Leeds, England). L-$^{14}$C(U)-arginine (330.0 mCi/mmol) was purchased from Du Pont NEN (Boston, MA).

Methods

Expression and Purification of nNOS - Heme-deficient apo-nNOS was expressed in Sf9 cells using a recombinant baculovirus as previously described (21). Sf9 cells were grown in SFM 900 II serum-free medium (Life Technologies, Inc.) supplemented with Cytomax (Kemp Biotechnology, Rockville, MD) in suspension cultures maintained at 27 °C with continuous shaking (150 rpm). Cultures were infected in log phase of growth with recombinant baculovirus at a multiplicity of infection of 1.0. After 48 h, cells were harvested and lysates prepared as previously described (21). Lysates from infected Sf9 cells (8 x 10$^9$) were centrifuged at 100,000 x g for 1 h. The supernatant fraction was loaded onto a 2'5'-ADP Sepharose column and the nNOS was affinity purified with the use of NADPH as described previously (3). Our initial studies, which are presented in Figs. 1 and 2, utilized this ADP-Sepharose purified preparation after 3 cycles of concentration and dilution with the use of a Centriplus concentrator with a 10,000 MW cutoff membrane (Amicon, Beverly, MA) to remove most of the NADPH. In all subsequent studies we utilized the following procedure to prepare the nNOS without added NADPH. We replaced NADPH with 10 mM 2’AMP in high salt buffer to elute nNOS from the 2'5'-ADP Sepharose column similar to that previously described (22). The nNOS containing fractions were combined and loaded onto a Sephacryl S-300 HR gel filtration column (2.6 x 100 cm, Pharmacia Biotech) equilibrated with 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10% glycerol, 0.1 mM EDTA and 0.1 mM DTT. The column was eluted at a flow rate of 1.3 ml/min and 1.5 ml-fractions were collected and analyzed for pro-
tein content and NOS activity. Fractions containing monomeric apo-nNOS were pooled and concentrated with the use of a Centriplus concentrator. The concentrated enzyme was aliquoted and stored at −80 °C. For preparation of purified heme-sufficient holo-nNOS, the same procedure was followed except oxyhemoglobin (25 µM) was added to the insect cells during expression.

Reconstitution of apo-nNOS – Sephacryl-purified apo-nNOS (2 µM) was added to a mixture containing 10 µM BH₄, 10 µM FAD, 10 µM FMN, 100 µM L-arginine, 200 u/ml catalase, 20 u/ml SOD, 1.5 mg/ml BSA, 100 mM KCl, 4 mM DTT in 10 mM Hepes, pH 7.4. Heme dissolved in DMSO was added to the mixture to give an 1.2-fold molar excess to that of nNOS. The final DMSO concentration was less than 2.2%. The mixture was incubated in the dark at room temperature. In the case of the nNOS that was purified only by ADP-Sepharose (Fig. 1 and 2), the reconstitution was as above except that the concentration of apo-nNOS was 3 µM.

Assays for NOS activity and ferrous carbonyl complex - NO synthesis activity was determined by measuring the conversion of methemoglobin to oxyhemoglobin. Aliquots (3 µl) of the reconstitution mixtures were added to an assay mixture containing 100 µM CaCl₂, 100 µM NADPH, 100 µM arginine, 100 µM BH₄, 100 u/ml catalase, 10 µg/ml calmodulin, and 25 µM oxyhemoglobin in a total volume of 180 µl of 50 mM potassium phosphate, pH 7.4. The mixture was incubated at 37 °C and the rate of oxidation of oxyhemoglobin was monitored by measuring the absorbance at λ₂₄₀–₄₁₁ nm with a microtiter plate reader as described (23). In some experiments, the NO synthesis activity was measured indirectly by determining the amount of conversion of ¹⁴C-labeled arginine to citrulline as previously described (21).

The ferrous carbonyl complex was measured with the use of an Aminco-DW2 spectrophotometer modified with the OLIS-DW2 operating system. An aliquot (175 µl) of the reconstitution mixture was added to 825 µl of 0.1 M potassium phosphate, pH 7.4, containing 20% glycerol, and 0.1 mM EDTA. CO was bubbled through the solution and a few grains of solid sodium dithionite were added. A spectrum was recorded and the concentration of ferrous-CO complex of nNOS was calculated using an extinction coefficient of 121 mM⁻¹ cm⁻¹ as previously reported (24).
Separation of nNOS monomer and dimer- nNOS monomer and dimer were separated by either HPLC gel filtration chromatography or low temperature SDS-PAGE. The SDS-PAGE procedure was similar to that described by Klatt et al. (25). Aliquots containing nNOS (6 µg) were quenched with ice-cold SDS sample buffer containing 6 mg/ml DTT, 100 µM BH₄ and 100 µM L-arginine. Samples were then resolved on 6 % SDS-polyacrylamide gels, which were kept cold during electrophoresis. Gels were stained with Coomassie Blue for detection of protein. nNOS monomer and dimer were also separated by the use of an HPLC gel filtration column (TSK G4000 SW, 7.5 x 300 mm, Toso Haas), Waters 600S controller, 717 plus autosampler, and 996 photodiode array detector (Waters Corp., Milford, MA). Aliquots (50 µl) of reconstitution mixtures were injected onto the column and subsequently eluted with ice-cold 50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 10% glycerol, 0.1 mM EDTA, 3 mM DTT, 10 µM BH₄ and 1 mM L-arginine at a flow rate of 1 ml/min. Absorbance was monitored at 280 and 400 nm.

Heme Assay - The amount of heme was determined by HPLC similar to that previously described (34). Samples were injected onto a C4 Vydac column (0.2 x 15 cm) equilibrated with solvent A (0.1% trifluoroacetic acid in water) at a flow rate of 0.3 ml/min. A linear gradient was started after 1 min to 30% solvent B (0.1% trifluoroacetic acid in acetonitrile) over 10 min. A linear gradient was then run to 55% and 100% solvent B over 35 and 3 min, respectively. In studies where radiolabeled heme was used, an on-line radiochemical detector (Radiomatic Flo-One) was used to measure the radioactivity. The recovery of radioactivity in each case exceeded 95%. Absorbance at 400 nm was monitored. Myoglobin (horse heart) was used as a standard for heme.
RESULTS

Effect of catalase and superoxide dismutase on the reconstitution of partially purified apo-nNOS - A partially purified preparation of apo-nNOS, which was prepared by ADP-Sepharose chromatography, was reconstituted with a 1.2-fold molar excess of heme and the NO synthase activity was measured. As shown in Fig. 1, the reconstitution of apo-nNOS with heme in the presence of arginine, BH₄, FMN, and FAD lead to an approximately 3-fold activation of apo-nNOS after 30 min to give a specific activity of 80 nmol of citrulline formed/min/mg of NOS protein (closed circles). This small increase in activity was lost after further incubation. The addition of SOD (open circles) gave a slight increase in activity whereas the addition of catalase (X) greatly enhanced as well as stabilized the activation. The addition of both SOD and catalase gave the greatest increase in activity (closed squares) giving a final specific activity of approximately 220 nmol of citrulline formed/min/mg of NOS protein, which is comparable to 224 nmol of citrulline formed/min/mg of NOS protein found for the ADP-Sepharose purified nNOS obtained from over-expression in heme-supplemented insect cells. The effect of SOD and catalase suggested a redox phenomenon involving the formation of hydrogen peroxide that somehow limited the insertion or stabilization of the heme-NOS complex. In support of this notion, as shown in Fig. 2, HPLC profiles of the mixtures after reconstitution of apo-nNOS with ¹⁴C-labeled heme clearly showed that the heme is destroyed during reconstitution in the absence of SOD and catalase (cf. panel A with panel B). The presence of SOD and catalase protected the heme from destruction (cf. panel C with panel B) and this effect is likely responsible for the enhanced and sustained activation of apo-nNOS. Since these results indicated damage to heme through a redox phenomenon during the reconstitution, we suspected that reducing agents were still present in this partially purified preparation. Thus, all subsequent studies on the reconstitution were performed with a preparation of apo-nNOS that was eluted with 2’5’-AMP instead of NADPH and further purified by Sephacryl gel filtration.
Reconstitution of Sephacryl-purified apo-nNOS - The reconstitution of the Sephacryl-purified apo-nNOS in the presence of SOD and catalase gave a time-dependent activation of NO synthase activity to a maximal level of approximately 1000 nmol of NO formed/min/mg of protein (Fig. 3A). BSA was also added to stabilize the heme. The NO synthase activity obtained after reconstitution is comparable to the 1100 nmol of NO formed/min/mg protein determined for Sephacryl-purified nNOS overexpressed from heme-supplemented insect cells, suggesting that we have succeeded in achieving maximal activation. As shown in Fig. 3B, there is a concentration dependence on apo-nNOS with values above 2 μM giving maximal activity. There is also a dependence on the concentration of DTT with values between 1 and 6 mM giving better activation, which is consistent with requisite coordination of heme to a cysteine thiolate (Fig. 3C).

We reexamined the components necessary for activation of apo-nNOS with the use of the Sephacryl preparation (Fig. 4). As expected, there was an absolute requirement for heme and DTT (lanes 2 and 3). The absence of BSA, catalase and SOD (lane 4) gave a decrease in the maximal activation from 1100 to 700 nmol of NO formed/min/mg protein, suggesting that we have minimized the redox destruction of heme but not abolished it. The omission of arginine (lane 5) or BH₄ (lane 6) also slightly decreased the activation.

Formation of the ferrous-CO complex during the reconstitution of apo-nNOS - As shown in Fig. 5, we examined the formation of the dithionite-reduced ferrous-CO complex during the reconstitution process. As expected, the heme-deficient apo-nNOS (lane 4) had low absorbance in the 400 to 450 nm region. After the addition of heme to apo-nNOS for 5 min and subsequent dithionite reduction in the presence of CO, an absorbance spectrum with maxima at 420 nm and 444 nm was observed (lane 2). The spectrum of heme in the same buffer but without apo-nNOS gave a peak at 420 nm (lane 1). Thus, the 444 nm peak is due to the ferrous-CO complex of NOS, which gave a calculated content of 0.58 mol of P450 per mol of monomer (inset). The ferrous-CO complex increased slightly to 0.65 mol of P450 per mol of monomer after 90 min of reconstitution (line 3 and inset). In that the formation of ferrous-CO complex occurred for the most part within 5 min, the slow increase in activity (Fig. 3A) cannot be due to slow heme insertion.
Formation of the homodimer of nNOS during reconstitution of apo-nNOS is concomitant with the increase in NO synthase activity - As shown in Fig. 6A, the formation of the dimeric form of nNOS was monitored by HPLC size exclusion chromatography. A sample containing a one-to-one mixture of purified apo-nNOS and heme-sufficient holo-nNOS (dashed line) showed a peak at an elution volume of 9.4 ml and 10.5 ml corresponding to the dimer and monomer, respectively. In studies not shown, each component was injected separately to confirm this assignment. The apo-nNOS reconstituted with heme for 5 min gave a broad peak centered at 10.3 ml representing mostly the monomeric form (dotted line). The apo-nNOS reconstituted for 120 min with heme eluted at 9.6 ml indicating that it was mostly dimeric (solid line). The absorbance of the peak corresponding to an elution volume of 9.4 ml was taken as a relative measure of the amount of dimeric nNOS that was formed and this absorbance value was plotted for samples of apo-nNOS reconstituted with heme over various durations (Fig. 6B). The increase in the amount of dimeric nNOS paralleled the slow increase in activity. Thus, it appears that the formation of the dimer is a slow process and is the rate limiting step in nNOS activation. We attempted to verify the amount of dimeric nNOS by low temperature SDS-PAGE analysis as previously described (26). As shown in Fig. 7, the Sephacryl purification of nNOS from heme-supplemented insect cells gave an intense Coomassie Blue-stained band at 320 kDa corresponding to the SDS-resistant dimer of nNOS. A less intense band at 160 kDa corresponding to the monomer of nNOS was also observed (lane 1). Interestingly, the in vitro reconstitution of apo-nNOS gave an intense band at 160 kDa but not at 320 kDa (lane 2). Thus, the dimeric form of nNOS formed in vitro is active, but is not SDS-resistant and is not the same species found in vivo.
DISCUSSION

In the current study, the monomeric heme-deficient apo-nNOS was functionally reconstituted to the level of fully active dimeric nNOS with the use of an in vitro system containing heme, BH₄, SOD, catalase, and arginine. SOD and catalase were necessary to remove reactive oxygen intermediates that formed during reconstitution and lead to heme destruction, which limited the activation of apo-nNOS as well as the stability of any activated nNOS that formed. Since catalase had a greater effect, it is likely that hydrogen peroxide was responsible, in large part, for alteration of the heme. The peroxidative degradation of free heme and cytochrome P450 heme has been described and is known to lead to ring-cleaved polar products (27). That polar heme degradation products are formed is consistent with the increase in polar radiolabeled products detected by reverse phase HPLC analysis of reaction mixtures after reconstitution of apo-nNOS with ¹⁴C-labeled heme in the absence of SOD and catalase. The source of the oxidants is likely redox reactions of heme with reductants, perhaps DTT, which was present in millimolar amounts in our experiments and is known to lead to heme degradation (28). Alternatively, redox reactions either by the flavin-reductase domain or heme domain of nNOS are known to produce superoxide, which can dismutate to hydrogen peroxide (29,30) and may cause heme degradation. However, these reactions should be slowed due to the absence of calmodulin or exogenously added NADPH in these studies. That oxidative damage can limit activation is consistent with the finding that partial reactivation of apo-nNOS was obtained in degassed buffers (14). Although it has been shown that NO inhibits heme insertion into iNOS in a macrophage cell line (6), our study suggests that for nNOS, reduced oxygen species may also serve to limit heme-mediated activation or reduce the stability of the enzyme.

Once we were able to achieve full activation of nNOS, we examined the kinetics of activation and its relationship to heme insertion and dimerization. We discovered that apo-nNOS activation is a slow process requiring over 90 min after heme addition to reach a maximum value whereas
heme binding to apo-nNOS was a rapid process going to completion in less than 5 min. The binding of heme to apo-nNOS was observed by the ability to form the ferrous-CO P450 complex, which indicated that the heme is bound to a cysteine thiolate. Since the NO synthase activity attains a maximal level only after 90 min, this form of nNOS P450 cannot synthesize NO. Moreover, this nNOS P450 was determined to be a monomer by size-exclusion chromatography and maybe similar to monomers obtained after urea treatment of dimeric iNOS (31,32). To our knowledge such a species has not been described for urea treatment of nNOS. We propose that this monomeric P450 species observed in our studies is an intermediate in the assembly of active nNOS dimers (Scheme I, Monomer$_2$). The slow activation of apo-nNOS was concomitant to the time course of dimerization of the heme-containing monomers and appears to be the rate-limiting step. The slow activation and dimerization is consistent with that found for iNOS (7) and is also consistent with the 4 h incubation needed to achieve partial activation of apo-nNOS, although the time course of activation of nNOS was not examined (14). In all cases, including the current study, the dimeric form of the NOS was observed by gel-filtration chromatography or by first employing a crosslinking reagent and subsequent analysis by SDS-PAGE (7,14). We unexpectedly found that the active dimeric form of nNOS that was formed in vitro is not SDS-resistant and could not be detected by low temperature SDS-PAGE, unlike that found for native nNOS dimers. Thus, we conclude that the dimer that is formed in vitro is not the same dimer as that formed in vivo even though both forms are able to synthesize NO (Scheme I, Dimer$_1$ and Dimer$_2$). Perhaps there are other post-translational events that regulate the formation of the stable SDS-resistant dimer, including chaperone-assisted folding, metal binding, or N-terminal domain swapping (10-12,17,21,33). At present we have not succeeded in conferring SDS-resistance in vitro with the use of reticulocyte extracts. It has been reported that BH$_4$ promotes the formation of SDS-resistant dimers of porcine brain NO synthase (25), however, we have not been successful in promoting formation of SDS-resistant dimers by adding BH$_4$ during reconstitution of monomeric nNOS. It is possible that BH$_4$ is necessary but not sufficient to cause dimerization of nNOS or that the observations with porcine NOS were due to stabilization of the SDS-resistant dimer that is already present but that normally
dissociates during the analysis.

The ability to fully activate apo-nNOS with near stoichiometric amounts of heme has at least two experimental uses; one to incorporate other porphyrin molecules to determine the effect of modified hemes on catalysis and second, to incorporate radiolabeled heme for use in inactivation studies. Mn and Co porphyrins have been incorporated into apo-nNOS but studies concerning their effects on catalysis have been precluded by the low activation observed by native heme (14). Our current study overcomes this problem. The incorporation of radiolabeled heme in nNOS would enable a more detailed study on the mechanisms of inactivation of the enzyme by various inactivators, including aminoguanidine (34) and \( \text{NO}^\text{2} \)-methyl-L-arginine (35), that are known to alter the heme prosthetic group. Although radiolabeled heme could be added during Sf9-mediated expression in insect cells, the *in vitro* reconstitution method is preferred since stoichiometric amounts of heme could be used. This method would be an alternative to the expression of NOS with radiolabeled delta-aminolevulinic acid to obtain NOS with radiolabeled heme (3).

Based on studies with iNOS and suicide inactivators, which inactivate only the functionally active enzyme, there appears to be a population of reserve monomeric iNOS in macrophage cells that can be recruited to form active dimers (36). Thus, the factors that govern this recruitment and assembly of dimeric NOS may be important in the effectiveness of these inactivators. In addition, our laboratory has shown that the level of nNOS protein is regulated in part by ubiquitination and proteasomal degradation (26,37) and may have *in vivo* consequences on nNOS levels (38). Moreover, the monomeric form of nNOS is preferentially ubiquitinated, suggesting that dimerization may play a role in stabilizing the protein *in vivo*. Thus, the mechanism involved in regulating the assembly of monomeric nNOS and stabilization of the dimeric form is likely to be important in regulating proteolytic removal of NOS as well as determining the rate of assembly of new active enzyme. The current study describes an *in vitro* system that allows for a more detailed exploration of the mechanism of reconstitution and activation of nNOS.
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FOOTNOTES

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1The abbreviations used are: NOS, nitric oxide synthase; nNOS, neuronal NOS; apo-nNOS, heme-deficient monomeric nNOS; iNOS, inducible NOS; SDS-PAGE, SDS polyacrylamide gel electrophoresis; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; HEK, human embryonic kidney; DTT, dithiothreitol; BH₄, (6R)-5,6,7,8-tetrahydro-L-biopterin; SOD, superoxide dismutase.
FIGURE LEGENDS

Fig. 1. **Reconstitution of heme-deficient apo-nNOS.** Apo-nNOS (3µM), which was purified by 2',5'-ADP-Sepharose chromatography with the use of NADPH, was reconstituted with heme as described in Experimental Procedures, except that BSA, catalase, and SOD were omitted (●). To this reconstitution mixture, 20 units/ml of SOD (O), 200 units/ml of catalase (X), or 20 units/ml of SOD and 200 units/ml of catalase (■) were added. Aliquots (10 µl) were taken for catalytic assays with radiolabeled arginine as described in Experimental Procedures. The specific activity of the arginine was recalculated to compensate for the addition of non-labeled arginine from the initial reaction mixtures. The specific activity of approximately 210 nmol of citrulline formed/min/mg of protein is comparable to 224 nmol of citrulline formed/min/mg found for the holoprotein after 2', 5'-ADP-Sepharose purification.

Fig. 2. **HPLC profile of apo-nNOS reconstituted with 14C-heme.** The conditions were as in Figure 1, except that 14C-labeled heme (132 mCi/mole, Leeds Radiochemicals) was substituted for heme. Aliquots (25 µl) were taken and analyzed for heme by HPLC as described in Experimental Procedures. A, starting heme; B, nNOS after reconstitution; C, nNOS after reconstitution in the presence of 20 units/ml of SOD and 200 units/ml of catalase.

Fig. 3. **Effect of time, concentration of apo-nNOS, and DTT on the reconstitution of Sephacryl-purified apo-nNOS.** A, apo-nNOS (2 µM), which was prepared by affinity purification with the use of AMP and gel filtration chromatography with the use of Sephacryl, was reconstituted as described under Experimental Procedures. Aliquots (3 µl) of the mixture were removed at various time points and assayed for NO synthesis activity by the oxyhemoglobin method. Values represent the mean ± S.E. from three separate experiments. B, apo-nNOS was reconstituted as above except that the concentration of apo-nNOS was varied while maintaining a 1.2-fold molar excess of heme. After incubation for 1.5 h, an aliquot (3 µl) was removed for measurement of NO
synthesis activity as in A. C, apo-nNOS (2 µM) was reconstituted as above for 1.5 h, except that DTT was either omitted or the concentration varied from 0.1 to 10 mM. An aliquot (3 µl) was removed for measurement of NO synthesis activity as in A.

Fig. 4. Components required for apo-nNOS activation. Apo-nNOS (2 µM) was reconstituted as described in Experimental Procedures (Lane 1). Lane 2, DTT was omitted; lane 3, heme was omitted; lane 4, catalase, SOD, and BSA were omitted; lane 5, arginine was omitted; lane 6, BH₄ was omitted. After incubation for 1.5 h, an aliquot (3 µl) of each mixture was removed and assayed for NO synthesis activity by the oxyhemoglobin method. Values represent the mean ± S.E. from three separate experiments.

Fig. 5. Formation of the ferrous-CO complex during reconstitution of apo-nNOS. Apo-nNOS (2 µM) was reconstituted as described in Experimental Procedures, except that FAD and FMN were not added. At various time points, aliquots (175 µl) were taken and the amount of ferrous-carbonyl complex was determined as described in Experimental Procedures. Line 1, reconstitution mixture without apo-nNOS; line 2, apo-nNOS reconstituted with heme for 5 min; line 3, apo-nNOS reconstituted for 90 min; line 4, apo-nNOS without added heme.

Fig. 6. Formation of dimeric nNOS during reconstitution of apo-nNOS. Apo-nNOS (2 µM) was reconstituted as described in Experimental Procedures, except that BSA was omitted. At various time points an aliquot (3 µl) was removed for analysis of NO synthesis activity and an aliquot (50 µl) was taken for analysis of dimer formation by the use of an HPLC TSK G4000 SW gel filtration column as described in Experimental Procedures. A, HPLC profile of nNOS monomer and dimer. The absorbance at 280 nm was measured. Apo-nNOS reconstituted for 5 min (dotted line), apo-nNOS reconstituted for 1.5 h (solid line), and a sample containing a mixture of apo-nNOS and heme-sufficient holo-nNOS (dashed line) purified from the insect cells are shown. In data not shown, the monomeric apo-nNOS and dimeric heme-sufficient holo-nNOS were injected.
individually and found to elute at 10.5 ml and 9.4 ml, respectively. B, apo-nNOS was reconstituted and aliquots (3 µl) were taken at the times indicated for determining the NO synthase activity (solid circles). Aliquots (50 µl) were also taken for HPLC analysis as in A. The absorbance at 280 nm for the fraction corresponding to the dimer (elution volume of 9.4 ml) was quantified (open circles). The data shown are representative of three separate experiments.

Fig. 7. In vitro reconstituted apo-nNOS is not SDS-resistant. Apo-nNOS (2 µM) was reconstituted for 1.5 h and an aliquot (23 µl) was taken for analysis of dimeric nNOS by SDS-PAGE as described in Experimental Procedures. Lane 1, holo nNOS purified from Sf9 cells; lane 2, reconstituted apo-nNOS. The gel was stained with Coomassie Blue. The gel shown is representative of three separate experiments.

Scheme I. Proposed model for stepwise assembly of nNOS. nNOS is assembled into its active form in a series of discrete steps. First, heme binds to the apo-nNOS (monomer₁) within 5 min and is coordinated as P450 type heme to form the monomer₂ species. Next, over the course of one to two h, two monomer₂ species associate to form dimer₁ and this homodimer is catalytically active. However, dimer₁ formed in vitro dissociates into monomers in the presence of SDS. An nNOS homodimer can be isolated from cells which is SDS-resistant (dimer₂), suggesting that further protein folding occurs in the cell to form this highly stable dimer. The term ‘active’ in this scheme refers to the ability to catalyze the formation of NO.
Figure 1
Figure 2

![Graph showing the measurement of 14C-heme (dpm) over time (min).](image-url)
Figure 3

A

B

C

nNOS Activity (nmol NO/min/mg)

Time (min)

nNOS (μM)

DTT (mM)
Figure 4

The figure shows a bar graph representing nNOS Activity (nmol NO/min/mg) across six conditions labeled 1 to 6. The graph indicates a significant increase in nNOS Activity from condition 1 to condition 4, followed by a decrease in conditions 5 and 6.
Figure 5

![Graph showing absorbance vs wavelength with P450 (mol/mol) values: apo 0.03, 5 min + heme 0.58, 90 min + heme 0.65.](image-url)
Figure 6

A

B
Figure 7
Scheme I

Monomer 1 (inactive) → Monomer 2 (inactive P450 heme) → Dimer 2 (active P450 heme, SDS-resistant)

Dimer 1 (active P450 heme)
Heme insertion, assembly, and activation of apo-neuronal nitric oxide synthase in vitro
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