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IMPROVED METHOD FOR ASSEMBLY OF HEMEPROTEIN NEURONAL NO-SYNTASE HETERODIMERS

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Abstract

The assembly of mutated and wild type monomers into functional heterodimeric hemeproteins has provided important mechanistic insights. As in the case of NO synthase (NOS), the existing methods to make such heterodimeric NOSs are inefficient and labor intensive with typical yields of about 5%. We have found that expression of neuronal NOS heterodimers in insect cells, where we take advantage of an exogenous heme-triggered chaperone-assisted assembly process, provides an approximately 43% yield in heterodimeric NOS. In contrast, in *E. coli* little heterodimerization occurred. Thus, insect cells are preferred and may represent a valuable method for assembly of other dimeric hemeproteins.

Keywords

nitric-oxide synthase; hemeprotein; site-directed mutagenesis; bacterial expression system; baculovirus expression system; homodimer; heterodimer

Mutational analysis is an important scientific tool but when proteins form multimeric complexes, the assembly of wild type monomers with specific mutant monomers is often desired. For example, in the case of dimeric hemeproteins, many important mechanistic questions have been answered by mutagenesis and reconstitution of heterodimers containing a wild type and a mutant monomer. For the dimeric hemeprotein nitric oxide synthase (NOS), assembling a truncated monomer with full-length mutant monomer to form heterodimers showed that the electrons from the flavin containing reductase domain of one monomer is transferred to the heme containing oxygenase domain of the other monomer in a “trans” fashion (1, 2). Better methods for the assembly of mutant heterodimers are needed as the current methods are inefficient and labor intensive requiring initial purification of the mutant homodimers, treatment with urea and detergent to obtain monomers, and subsequent removal of the denaturant to allow for heterodimerization (1, 2). For neuronal NOS (nNOS) the initial step of monomerization was approximately 30% effective whereas the use of

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inducible NOS (iNOS) mutants that favor monomerization increased this to 70–90% (1, 2). However, even with these iNOS mutants, the overall yield of obtaining heterodimers was approximately 6% (3). In the current study, we explore recombinant expression of heterodimers in *E. coli* and Sf9 insect cells to improve that of the established *in vitro* methods utilizing purified proteins (1, 2).

To perform these studies, we took advantage of the known *trans* delivery of electrons and specific site-directed mutants of nNOS such that only the heterodimer, but not homodimers, would have activity (Fig. 1).

As will be described in detail later, we made site-directed mutants that inactivate the oxygenase domain (E592A, Oxy-dead mutant, Fig. 1 *form a*) or reductase domain (S1176A, Red-dead mutant, Fig. 1 *form b*). The homodimers of these mutants will be inactive and only when the mutants form heterodimers and electron flow can occur through a *trans* mechanism will the protein have NO synthase activity (Fig. 1, *form c*). These residues were chosen based on established precedent (1, 2, 4). Moreover, we placed either a His₆- or FLAG-tag on each of the mutants so that we could monitor the amounts of each mutant expressed under the different experimental conditions.

More specifically, to over-express His₆- and FLAG-tagged mutant nNOS, the nNOS gene was modified and subcloned to either pCWori or pFastBac/CT-TOPO vectors. The cDNA of the nNOS WT was obtained from the pCWnNOS-WT plasmid previously used in our laboratory where the nNOS gene was inserted between the NdeI and NotI restriction sites (5). To insert the His₆- or FLAG-tag to the C-terminus of the nNOS WT, PCR was performed using the pCWnNOS-WT plasmid as the DNA template and a pair of PCR primers in the presence of dNTPs and Phusion High-Fidelity polymerase (Thermo Fischer Scientific). The NdeI_S/Histag_AS and NdeI_S/Flagtag_AS pairs of primers were used to introduce a 6xHis-tag and FLAG-tag, respectively. (NdeI site:

GCTTAGGAGGTCATATGGAAGAGAACACGTTTGG; His₆-tag:

CTGCGGCCGCTTAGTGGTGATGGTGGTGATGGGAGCTGAAAACCTCATC; FLAG-tag:

CTGCGGCCGCTTATTTATCGTCATCATCTTTATAATCGGAGCTGAAAACCTCATC).

The 4.3-kb PCR products were then digested with a pair of NdeI/NotI restriction enzymes and then ligated to the 6-kb DNA fragment of the pCWnNOS-WT digested with the same pair of restriction enzymes. New constructs of the plasmids containing the His₆- or FLAG-tag were selected from single colonies of the DH5α cells transformed with the ligated products in the presence of ampicillin as an antibiotic marker. The new constructs containing the His₆- and FLAG-tag are referred to as pCWnNOS6xHis_WT and pCWnNOSFlag_WT respectively. The mutant nNOS, E592A and S1176A, were constructed by the QuikChange method (Thermo Fischer Scientific) according to the manufacturer's protocol using a pair of complimentary mutagenic primers (E592A_S:

GGTACATGGGCACAGCGATCGGCGTCCGTGACTAC; E592_AS:

GTAGTCACGGACGCCGATCGCTGTGCCCATGTACC; S1176A_S:

CTGCAGCCTCGCTACTACGCGATCAGCTCCTCTCCAG; S1176A_AS;

CTGGAGAGGAGCTGATCGCGTAGTAGCGAGGCTGCAG).

The nNOS mutants were expressed in *E. coli*, purified, and the activity measured by the oxyhemoglobin method as previously described (5, 6). As shown in Fig. 2A, we individually expressed the homodimers in *E. coli* the Oxy-dead nNOS mutant (*form a*) or the Red-dead nNOS mutant (*form b*) and verified that the cytosolic preparations as well as the purified preparations exhibited little or no nNOS activity. For *in vitro* heterodimerization of nNOS mutants, we followed the method of Sagami *et al.* (2) where the purified homodimeric nNOS was dissociated into monomers in the presence of urea and detergent, mixed with an equal amount of the other similarly monomerized mutant nNOS, and subsequently diluted with buffer containing substrate and tetrahydrobiopterin. The resulting reconstituted mixture contains some heterodimer as evident by nNOS activity of approximately 5 nmol/min/nmol P450 (Fig. 2A, *form c*). We next tried assembly in *E. coli* cells by co-expression of the mutant nNOS forms. We obtained a strain that expressed approximately equal amounts of each mutant as detected by Western Blot (Fig. 2B, *lane 2*). The crude cytosol from these cells gave an activity of 4 nmol/min/nmol P450 (Fig. 2A, *form c*). Thus, *in vitro* reconstitution or co-expression in *E. coli* both gave relatively low levels of heterodimers, consistent with previous reports (1, 2). These values are low in comparison to the activity of the purified wild-type nNOS of 138 ± 11 nmol/min/nmol P450 ($n=5$) and the FLAG-tagged wild type nNOS of 145 ± 23 nmol/min/nmol P450 ($n=5$). The FLAG-tag did not appear to have an effect on the activity.

We thought that expression of the heme proteins in insect cells, which is known to produce heme-deficient apo-protein that can then be reconstituted with exogenous heme, may provide a better way to make heterodimers. We expressed approximately equal quantities of mutant nNOS in Sf9 cells (Fig. 2B, *lane 3*) and subsequently added heme to the intact cells as previously described (6). We have shown previously that the heme is inserted into the apo-nNOS by a chaperone-dependent process and triggers the assembly of nNOS dimers (6). This procedure gave an activity of 38 nmol/min/nmol of P450 (Fig. 2A, *form c*). This is approximately an 8- to 10-fold increase in heterodimerization efficiency over the published methods as well as that described in the current study using bacterial expression system.

To better estimate the amount of heterodimer formed, we purified the heterodimeric mutant nNOS (*form c*) by FLAG- and Ni-chromatography and the resulting preparation has an activity of 88 ± 10 nmol/min/nmol P450 ($n=3$). This would be the theoretical maximum activity if the preparations were fully heterodimeric. Thus, based on the activity of the purified heterodimeric mutant nNOS (*form c*), we estimate the extent of heterodimerization in bacteria and insect cells to be approximately 4% and 43%, respectively.

To further verify that heterodimer formed, we took the active insect cytosol containing equal amounts of FLAG- and His₆-tagged nNOS and immunoprecipitated with anti-FLAG to bring down *forms a* and *c*. As shown in Fig. 2C, there was nearly complete disappearance of FLAG from the supernatants (*squares*) whereas about a third of the His₆-tagged signal was immunoprecipitated (*circles*). As a control, we determined that the anti-FLAG did not immunoprecipitate the His₆-tagged dimer (*Form b, triangles*). Thus, we have qualitative evidence that heterodimeric mutant nNOS (*form c*) was formed. We have performed the same pull-down study with the bacterial cytosol and found no heterodimeric mutant nNOS

(*form c*) being pulled down by the anti-FLAG antibody (data not shown), consistent with the estimate of 4% heterodimer based on activity measurements.

In conclusion, we have utilized site-directed mutants of nNOS that readily allow the comparison of different methods for heterodimerization. Since the homodimers containing the oxygenase mutation nor the homodimers containing the reductase mutation do not have activity whereas the heterodimers do, we can very easily compare the heterodimerization efficiency among *in vitro*, *E. coli*, and Sf9 systems by measuring the nNOS activity of the mixtures of purified proteins or in crude cytoplasmic preparations prepared from the cells. We found that expression in insect cells and subsequent triggering of the assembly of the hemeproteins into heterodimers is at least 8-fold more efficient than the existing methods. Not only is there greater efficiency of the heterodimerization, there is no need to purify the mutant homodimers for subsequent monomerization and heterodimerization. We believe this procedure will likely work with other dimeric hemeproteins. Interestingly, in the case of *E. coli* expression, where there is abundant endogenous heme to allow for efficient synthesis of the heme-containing holo-nNOS, the assembly of heterodimers is inefficient perhaps due to the efficient incorporation of heme and assembly of homodimers soon after synthesis.

Acknowledgments

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Abbreviations

NOS	nitric-oxide synthase
nNOS	neuronal NOS
Oxy	oxygenase
Red	reductase

References

1. Panda K, Ghosh S, Stuehr DJ. J Biol Chem. 2001; 276:23349–56. [PubMed: 11325964]
2. Sagami I, Daff S, Shimizu T. J Biol Chem. 2001; 276:30036–42. [PubMed: 11395516]
3. Siddhanta U, Wu C, Abu-Soud HM, Zhang J, Ghosh DK, Stuehr DJ. J Biol Chem. 1996; 271:7309–12. [PubMed: 8631749]
4. Panda SP, Gao YT, Roman LJ, Martasek P, Salerno JC, Masters BSS. J Biol Chem. 2006; 281:34246–57. [PubMed: 16966328]
5. Clapp KM, Peng HM, Morishima Y, Lau M, Walker VJ, Pratt WB, Osawa Y. J Biol Chem. 2010; 285:33642–51. [PubMed: 20729196]
6. Billecke SS, Draganov DI, Morishima Y, Murphy PJM, Dunbar AY, Pratt WB, Osawa Y. J Biol Chem. 2004; 279:30252–8. [PubMed: 15155759]
7. McMillan K, Masters BSS. Biochemistry. 1995; 34:3686–93. [PubMed: 7534476]

Forms a b c

Fig. 1. Schematic representation of the mutant nNOS forms used in these studies

Form a, homodimeric nNOS containing FLAG-tagged nNOS E592A (Oxy-dead mutant denoted by a star in circle representing the oxygenase domain of nNOS); *form b*, homodimeric nNOS containing His₆-tagged nNOS S1176A (Red-dead mutant denoted by a star in rectangle representing the reductase domain of nNOS); *form c*, heterodimeric nNOS containing a monomer of FLAG-tagged nNOS E592A (Oxy-dead mutant) and a monomer of His₆-tagged nNOS S1176A (Red-dead mutant).

Fig. 2. Activities and amounts of the homodimeric and heterodimeric forms of nNOS generated in these studies

A, the experimentally observed nNOS activities of *forms a, b, and c* from purified preparations, *E. coli* cytosol, and Sf9 insect cytosols are shown. Activities are expressed per spectrally observed ferrous-CO P450 complex (7). Purified proteins were used in the *in vitro* reconstitution conditions and the activity is on the resulting mixture. The various forms were expressed in *E. coli* or Sf9 insect cells and the cytosolic fraction was prepared for activity measurements. The values are the mean ± SE of triplicate samples. **B**, equal amounts of His₆- and FLAG-tagged nNOS were used in *in vitro*, *E. coli*, and Sf9 conditions as determined by Western blotting. *Lane 1*, reconstituted purified proteins (0.5 μg); *lane 2*, cytosol from *E. coli* expression (15 μg); *lane 3*, cytosol from Sf9 expression (7.5 μg). Samples were immunoblotted with 0.4 μg/ml of rabbit anti-FLAG IgG (SIGMA F7425) and IRDye-conjugated goat anti-rabbit IgG (Li-Cor) at 1:25,000 dilution or with 1 μg/ml of mouse anti-His₅ IgG (QIAGEN 34660) and IRDye-conjugated goat anti-mouse IgG (Li-Cor) at 1:10,000 dilution. **C**, amount of heterodimer *form c* in the active insect cytosols determined by immunoprecipitation. Fifty μg of cytosol of Sf9 cells containing homodimer *form b* (triangles) or heterodimer *form c* (squares and circles) were incubated at 4 °C for 1.5 h in a total volume of 100 μl with mouse monoclonal anti-FLAG IgG-coupled agarose

(SIGMA A2220). Five % of the supernatant fraction was Western blotted as in *B*. The signals of nNOS bands were quantitated with Image Studio (Li-Cor). The supernatants from anti-FLAG immunoprecipitates were blotted with anti-FLAG (*squares*) and anti-His₅ (*circles* and *triangles*). The values are the mean $\pm$ SE of triplicate samples.

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