Published in final edited form as:

J Biol Inorg Chem. 2019 February ; 24(1): 1–9. doi:10.1007/s00775-018-1621-1.

Generation and Characterization of Functional Phosphoserine Incorporated Neuronal Nitric Oxide Synthase Holoenzyme

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Abstract

Phosphorylation is an important pathway for regulation of nitric oxide synthase (NOS) at the posttranslational level. However, the molecular underpinnings of NOS regulation by phosphorylations remain unclear to date, mainly because of the problems in making a good amount of active phospho-NOS proteins. Herein, we have established a system in which recombinant rat nNOS holoprotein can be produced with site-specific incorporation of phosphoserine (pSer) at residue 1412, using a specialized bacterial host strain for pSer-incorporation. The pSer1412 nNOS protein demonstrates UV-vis, far UV CD and fluorescence spectral properties that are identical to those of nNOS overexpressed in other bacterial strains. The protein is also functional, possessing normal NO production and NADPH oxidation activities in the presence of abundant substrate L-Arg. Conversely, the rate of FMN – heme interdomain electron transfer (IET) in pSer1412 nNOS is considerably lower than that of wild type (wt) nNOS, while the phosphomimetic S1142E mutant possesses similar electron transfer kinetics to that of wt. The successful incorporation and high yield of pSer1412 into rat nNOS and the significant change in the IET kinetics upon the phosphorylation demonstrate a highly useful method for incorporating native phosphorylation sites as a substantial improvement to commonly used phosphomimetics.

Keywords

nitric oxide synthase; phosphoserine; phosphorylation; electron transfer; phosphomimetic

Introduction

The nitric oxide synthases (NOSs) comprise a family of enzymes catalyzing the oxidation of L-arginine (L-Arg) to citrulline and nitric oxide (NO) [1, 2]. There are three mammalian NOS

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isoforms: endothelial, neuronal, and inducible NOS (eNOS, nNOS, and iNOS, respectively). Each subunit of the NOS homo-dimer has two domains joined by a calmodulin (CaM) binding linker: a C-terminal electron-supplying reductase domain, which consists of (sub)domains with binding sites for NADPH (the electron source), FAD, and FMN, and an N-terminal catalytic heme-containing oxygenase domain. The substrate L-Arg and a cofactor, (6R)-5,6,7,8-tetrahydrobiopterin (H_4B), bind near the heme center in the oxygenase domain. The CaM-binding linker in iNOS binds CaM at basal levels of Ca^{2+} , while in nNOS and eNOS the CaM binding requires an increase in intracellular $[Ca^{2+}]$ [3]. In addition to control via Ca^{2+}/CaM , eNOS & nNOS are regulated by posttranslational modifications including phosphorylation at several sites. The functional importance of NOS phosphorylation has been established *in vivo* [4, 5]. Such a complex set of modulators provides stringent regulation in both time and place for NO production in response to a wide variety of stimuli.

Mechanisms that explain the effects of phosphorylation at specific residues such as serine on NOS activity have been extensively investigated using purified phospho-proteins [6] and phosphomimetic mutants [7-10]. Phosphorylated NOSs are difficult to purify in adequate amounts because the phospho-protein levels are low *in vivo*. *In vitro* phosphorylation by a kinase can be complicated by the facts that the kinase may not just phosphorylate a single site, and that the NOS protein may become less stable after long incubation at room temperature. In a common phosphomimetic approach, the phosphorylated residue of interest is mutated to a negatively charged residue, either aspartate or glutamate, imparting the negative charge associated with phosphorylation (Figure 1). However, this amino acid approximation approach has significant limitations that include a change in negative charge on the aspartate/glutamate substitution from that of the phosphorylated residue at physiological pH, and a different overall chemical environment from that created by phosphorylation. Notably, the pK_a values of the carboxylate group of aspartate and glutamate (1.88 and 2.19, respectively) are different from those of a phosphate group (e.g., 5.6 for phosphoserine [11]). Since the phosphomimetic mutant cannot fully recapitulate the electrostatic and structural nature of the phosphorylation modification and does not accurately represent phosphorylation, there are fundamental concerns regarding how these limitations may impact the interpretation of findings in studies using these phosphomimetic mutants. In fact, observed mutational effects are often controversial [12]. A recent finding further showed that mutation of Thr to Asp cannot fully mimic the effect of phosphorylation on activities of eNOS [13]. Thus, the molecular mechanisms underlying NOS regulation by phosphorylations remain unclear to date.

To overcome this critical barrier in studying phosphorylated NOSs, in the present work we have utilized a new synthetic biology method [14] to site-specifically incorporate phosphoserine (pSer) at residue 1412 in the C-terminal tail of rat holo-nNOS. We chose this site because its phosphorylation plays a critical role for *in vivo* control of nNOS [15]. Herein we have demonstrated successful incorporation and high yield of pSer1412 into rat nNOS and the significant change in the rate of the FMN – heme interdomain electron transfer upon the phosphorylation. This is a highly useful method for incorporating native-like phosphorylation sites as a substantial improvement to commonly used phosphomimetics.

Materials and Methods

Construction of pSer-incorporation plasmids.

Rat holo-nNOS expression plasmid (pJH-rnNOS) was constructed using circular overlap extension PCR. Backbone PCR product was amplified from plasmid pCRT7 NT Topo tetR/pLtetO Amp-wt sfGFP (Addgene # 52053) [14] and the rat nNOS gene was amplified from a N-terminal His-tagged wild type nNOS plasmid. The two fragments were circularized with overlap sequences. S1412TAG-nNOS expression plasmid (pJH-rnNOS S1412TAG) was then constructed from pJH-rnNOS using site-directed mutagenesis. TAG codon installation at 1412 was verified by sequencing.

pSer1412 nNOS protein expression and purification.

The C321. A cells [14] harboring pJH-rnNOS S1412TAG plasmid with SepOTS λ were transferred to 5 mL of LB media containing 25 μ g/mL kanamycin, 100 μ g/mL ampicillin, and 0.08% glucose and grown overnight at 250 rpm. Five hundred mL of TB media containing 25 μ g/mL kanamycin, 100 μ g/mL ampicillin, 0.08 % glucose and 5 mM o-phospho-L-serine was inoculated with the resulting saturated cell culture and grown at 30 °C, 250 rpm to OD600 of 0.8. Induction cocktails containing 1 mM IPTG and 100 ng/mL anhydrotetracycline, 75 mg/L δ -ALA and a pinch riboflavin were added to the TB culture and the resulting culture was then switched to grow at 24 °C, 250 rpm, for 40 h. The cells were harvested and stored at -80 °C. Protein purification was performed as described [16]. After sonication, the lysate was applied to a 2',5'-ADP Sepharose affinity column equilibrated in binding buffer, and the nNOS protein was eluted with NADP disodium.

Determination of the extent of phosphorylation of purified pSer1412 nNOS.

The pSer incorporation was determined using the Phosphoprotein Phosphate Estimation Assay Kit (Thermo Scientific). The manufacturer's protocol was modified by adding 0.2% polyvinyl alcohol (PVA) to minimize the precipitation of the malachite green-phosphomolybdate complex [17, 18]. Briefly, 52 μ g wt or pSer1412 nNOS and the phosphitin standard were diluted with 0.2 mL of TBS, incubated with 0.2 mL of 2.0 N NaOH at 65 °C for 30 min, and centrifuged at 12,000 g for 3 min after adding 0.2 mL of 4.7 N HCl. The supernatant was transferred into a new tube, and PVA was added to a final concentration of 0.2 %. After 30 min incubation at room temperature in dark, the absorbances of each standard and sample at 625 nm were measured on a Cary 50 UV-vis spectrophotometer. Lambda Protein Phosphatase (Lambda PP, New England Biolabs) was further used as a negative control to dephosphorylate both the wt and pSer1412 nNOSs: the wt and pSer1412 nNOSs were each incubated with 1000 units Lambda PP in 1X Reaction Buffer with added 1 mM MnCl₂ at 30 °C for 30 min. After centrifugation in a 100 K cutoff centrifuge tube, the sample was exchanged into TBS for phosphate estimation assay. The obtained number of phosphate per nNOS subunit n is listed in Table S1.

Protein phosphorylation identification by LC-MS/MS.

Mass spectrometry data were acquired at the University of Arizona Analytical and Biological Mass Spectrometry Facility. Gel slices were digested with trypsin (Pierce

Biotechnology, Rockford, IL) for 3 hours at 37°C using ProteaseMax™ Surfactant trypsin enhancer following reduction and alkylation with dithiothreitol and iodoacetamide, respectively, according to the manufacturer's instructions (Promega Corporation, Madison, WI). The peptides were desalted using Carbon NuTips (Glygen Corp, Columbia MD). LC-MS/MS analysis was carried out using a Q Exactive Plus mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with a nanoESI source (see Supporting Information for details in the MS/MS data analysis).

Construction and preparation of S1412E rat nNOS mutant.

Rat holo-nNOS S1412E mutation was created using a nested PCR technique. Primers were designed to amplify the regions of recombinant nNOS from the start to the mutation (PCR #1), and from the mutation to the STOP codon (PCR #2). Products from these amplifications were then used as template for a third PCR reaction (PCR #3), spanning the entire protein sequence. The resultant product is an NOS coding sequence with the indicated mutation. The PCR products from PCR #3 were restriction digested with NdeI/XbaI (nNOS) and ligated with NdeI/XbaI-digested pCW vector. This ligation was used to transform XL10-gold cells (Stratagene) and colonies were screened by restriction digest. The correct construct was then used to transform *E. coli* BL21 cells. Rat nNOS S1412E was grown and purified as described [16].

NOS enzymatic activity assays.

Steady-state rates of NO production by nNOS were determined in pH 7.4 buffer containing 50 mM Tris, 100 mM NaCl, 5 μ M H₄B, and 200 μ M CaCl₂ [16]. Sample solutions contained 100 μ M L-Arg, 100 μ M NADPH, 8 μ M oxyhemoglobin and 1 μ M CaM. Reactions were initiated by adding nNOS protein (20 nM final concentration), and rate of NO synthesis was determined using extinction coefficients of 60 mM⁻¹ cm⁻¹ at 401 nm. Oxidation of NADPH was monitored at 340 nm under same conditions, and the rate was determined using an extinction coefficient of 6.2 mM⁻¹ cm⁻¹.

CD spectroscopy.

Room-temperature CD spectra were recorded on a JASCO J-810 spectropolarimeter employing a Hamamatsu photomultiplier tube, using a 0.1-cm path-length quartz cell (Hellma USA Inc., Plainview, NY, USA). The instrument was calibrated using CSA, (1S)-(+)-Camphor-10-sulfonic acid, 1 mg/ml in a 0.1 cm cell [19]. Protein samples were diluted into phosphate buffer to 0.1 μ M and filtered through a 0.2 μ m filter. CD Spectra and buffer baselines were averaged from 16 scans, each recorded at 0.5 nm data pitch, using a scanning rate of 50 nm/min and a response of 0.125 s. Subtracting the buffer baseline from samples yields the net CD spectra solely from the protein.

Fluorescence spectra of NOS.

The NOS flavin fluorescence spectra were measured on a Cary Eclipse Fluorescence Spectrophotometer (Agilent Technologies) at room temperature. nNOS protein in buffer was filtered by a 0.2 μ m membrane filter. A quartz cuvette with 1 cm path length was used for

the experiments. The sample was excited at 446 nm, and the fluorescence spectra were recorded from 480 to 700 nm.

Laser flash photolysis.

The FMN – heme interdomain electron transfer kinetic measurements were conducted on an Edinburgh LP920 laser flash photolysis spectrometer, in combination with a Q-switched Continuum Surelite I-10 Nd:YAG laser and a Continuum Surelite OPO. The CO photolysis experiments were performed as described [20]. Briefly, sample contained ~ 20 μ M dRF and 5 mM fresh semicarbazide in a pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μ M H₄B, 1 mM Ca²⁺ and 10 % glycerol). Sample without protein in a 1 cm cuvette sealed with a rubber septum was deaerated by bubbling with mixed CO/Ar (v/v 1:3) gas for 1 h. The gas was blown over the sample surface to remove traces of O₂ added upon subsequent introduction of nNOS protein aliquots to the sample. The pre-degassed sample was illuminated for a certain period of time to obtain a partially reduced form of [Fe(II)–CO][FMNH[•]]. The sample was then flashed with 446 nm laser excitation to trigger the FMN – heme IET, which can be followed by the loss of absorbance of Fe²⁺ at 460 nm [21]. The experiments were conducted at least twice. The transient absorbance changes were averaged and analyzed using OriginPro 9.0 (OriginLab, Northampton, MA, USA).

Results

Generation of the phosphoserine 1412 incorporated rat nNOS holoprotein.

To examine the performance and compatibility of the phosphoserine incorporation plasmid, *E. coli* C321. A was co-transformed with pJH-rnNOS or pJH-rnNOS S1412TAG with SepOTS λ (Addgene # 68292). *E. coli* C321. A is a specialized bacterial host strain for pSer-incorporation [14]. Small scale nNOS purification was done via nickel-NTA affinity chromatography. The isolated proteins were subjected to anti-His and anti-phospho-nNOS (S1412) immunoblot analysis. The anti-His results (Figure S1) show that C321. A harboring pJH-rnNOS or pJH-rnNOS S1412TAG with SepOTS λ grown in the presence of 2 mM of o-phospho-L-serine (Sep) produced rat nNOS or amber suppressed nNOS, respectively; Sep and p-Ser are used interchangeably as abbreviation of phosphoserine. To confirm the incorporation of Sep into nNOS protein at residue 1412, the phospho-nNOS (Ser 1412) polyclonal antibody (PA1-032, Thermo Fisher Scientific) was used. The result (Figure S1, antiphospho-nNOS, lane 2) clearly shows that Sep-incorporated nNOS at residue 1412 is produced in the C321. A harboring SepOTS λ with pJH-rnNOS S1412TAG grown in the presence of 2 mM Sep; to further improve yield of pSer1412 nNOS, 5 mM Sep was used for large scale overexpression of the protein.

Rat nNOS with S1412 mutated to a TAG codon was co-expressed with SepOTS λ in *E. coli* C321. A [14] and purified to generate pSer1412 nNOS. We showed that the pSer1412 nNOS was successfully overexpressed in *E. coli* C321. A (Figure 2A & 2B and Figure S1), with an average yield of 6 mg of isolated enzyme per liter of cells. The site-specific pSer incorporation at 1412 has been confirmed by MS/MS analysis (Figure 2C). To our knowledge, this is the first report on high-level production of pSer-incorporated NOS. It is important to note that robust incorporation of p-Ser into proteins has not become possible

until recently [14, 22], and that the applications of such an approach are still limited to rather small proteins [23, 24], while nNOS has a molecular weight of ~ 320 kDa for the homodimer. As expected, wild type (wt) nNOS expressed in C321. A is non-phosphorylated at residue 1412 (Figure 2A).

To determine the pSer incorporation efficiency, we measured the level of phosphate anion liberated from the nNOS proteins by hydrolysis in the presence of 0.2% polyvinyl alcohol (PVA) to minimize the precipitation of the malachite green-phosphomolybdate complex [17, 18]. The formation of the soluble green complex formed among malachite green, molybdate, and free orthophosphate can be readily monitored by absorbance at 625 nm. We observe that there is a significant difference in the phosphate number of the wt and pSer1412 nNOSs (Table S1). The phosphate level in the wt protein is not zero, and the value of pSer1412 nNOS decreased notably after the phosphatase treatment (Table S1). The absorbances at 625 nm of both the wt and pSer1412 nNOSs after the phosphatase treatment were still higher than the background absorbance of the assay buffer, which may be due to phosphorylation at Tyr residue(s) and/or residual phosphorylations at Ser/Thr site(s) because Lambda PP has greater reactivity against phospho-serine/threonine residues. These phosphatase treatment results confirmed that there are indeed basal phosphorylations in the wt protein. Based on the immunoblot analyses (Figure 2A) and the significant difference in phosphate levels of pSer1412 and wt nNOSs (Table S1), the pSer1412 incorporation level can be estimated to be ~ 70% by subtracting the 'net' value of wt from that of the pSer1412 (Table S1). This estimation is further supported by the significantly different FMN – heme interdomain electron transfer (IET) kinetics (see below).

Our mass spectrometric (MS) analyses of the wt protein further showed that there are three phosphoserines and one phosphothreonine (Figure S2). Such background phosphorylations in recombination wt nNOS have also been observed for nNOS protein overexpressed in BL21(DE3) cells (Figure S3). In other words, the 'basal' phosphorylations are not caused by the different *E. coli* strain used in this work. Background phosphorylations in recombinant proteins overexpressed in *E. coli* have been recently reported for other proteins [25, 26]. However, we note that these background phosphorylation sites in the wt nNOS are not at any known regulatory regions (Figure S4). The LC-MS/MS method can qualitatively identify the phosphorylation site and type, but is not yet a reliable approach to assess phosphorylation stoichiometry due to different ionization efficiencies of phospho- and unmodified peptides [27]. Therefore, we did not attempt to quantify the phosphopeptides in the wt and pSer1412 nNOS samples.

Measurement of enzymatic activities of the pSer1412 rat nNOS protein.

To confirm that the isolated proteins are active, we determined NO production and NADPH oxidation rates (Table 2). Under optimal fully coupled conditions, NO production by NOS requires 1.5 NADPH molecules per NO molecule formed. Deviations from this optimum value indicate that reactive oxygen species are being formed at the expense of the NO product. The NO synthesis rate ($1.21 \pm 0.12 \text{ s}^{-1}$) of wt nNOS expressed in *E. coli* C321. A is in the range of prior literature values [16]. Within experimental error, there is no significant difference in the rate of NO formation between the wt and pSer1412 nNOS

proteins (Table 1): unpaired *t*-test gives the two-tailed *P* value of 0.4319; by conventional criteria, this difference is considered to be not statistically significant. Moreover, the NADPH/NO ratio for the pSer1412 and wt nNOS protein in the presence of saturated L-Arg is 2.3 ± 0.3 and 2.6 ± 0.4 (Table 2), giving the two-tailed *P* of 0.3574 in *t*-test; this difference is not statistically significant, either.

This observation is in contrast with a previous kinetic study of the phosphomimetic S1412D nNOS mutant, which reported a much higher NADPH/NO ratio (3.65) for the mutant, compared to the purified wt protein [9]; a 25% diminished NO production activity was reported for this S1412D mutant [9]. Conversely, the NO synthesis rate of wt and S1412E nNOS proteins expressed in *E. coli* strain BL21(DE3) is 0.94 ± 0.03 and $0.83 \pm 0.01 \text{ s}^{-1}$, respectively, showing a much smaller (10%) decrease in the activity of the S1412E mutant (Table 1). Glutamate is considered to be a better mimic of pSer than aspartate because Glu is similar in size to pSer (Figure 1). What phosphorylation of S1412 does *in vivo* remains a controversial topic. There are reports of increased NO production [28, 29], while other studies observed no increase upon the phosphorylation [30, 31]. To address this issue, our *E. coli* produced pSer-incorporated nNOS will allow these possibilities to be tested *in vitro*.

Spectral characterizations of the pSer1412 rat nNOS protein.

We next characterized the spectral properties of purified pSer1412 holo-nNOS. Absorption spectra of as-isolated pSer1412 nNOS protein are shown in Figure S5. The heme site is predominantly in the high spin state after treatment with H₄B and L-Arg during preparation, as evidenced by the Soret peak at 393 nm. Shoulders are also apparent at 450 and 475 nm that are due to flavin absorbance. Based on the CO difference spectrum (inset of Figure S5), the heme center is in the native thiolate-heme complex absorbing at 444 nm, with no detectable 420 nm band. Collectively, these data reflect the intactness of the cofactor and substrate binding sites, as well as the accessibility of sites to H₄B and L-Arg.

To probe secondary structure of the purified recombinant nNOS proteins, far UV CD spectra were collected in the 190 – 260 nm wavelength range (Figure 3). The well-defined spectra are characterized by minima at 208 and 222 nm, a maximum at 192 nm and baseline cross-over point at 200 nm. Virtually identical spectra were obtained for the wt and pSer1412 nNOS proteins (Figure 3), and the spectra also match those of nNOS isolated in other laboratories [32, 33]. Therefore, the wt protein overexpressed with the new vector in *E. coli* C321. A strain folds properly, and pSer incorporation does not appear to have caused any significant structural perturbations, compared to the wt protein. This is consistent with the location of the 1412 residue, which is in the C-terminal tail (CT), where pSer1412 incorporation is not expected to perturb the overall folding of the nNOS protein.

Flavins bound to a protein can exhibit either enhanced or quenched fluorescence to a degree that is both specific and characteristic of the particular protein. The FMN fluorescence intensity of nNOS is known to be influenced by CaM binding in a partially reversible manner [34]. This is attributed to a CaM binding-triggered conformational change, in which the FMN and FAD domains are separated, leading to further exposure of the FMN to solvent and a large increase in the FMN fluorescence. The FMN fluorescence spectra of the nNOS proteins were collected under different calcium concentrations. Fluorescence of pSer1412

nNOS was increased by a factor of 2.7 after Ca^{2+} -promoted CaM binding to nNOS, and the increase was partly reversed by dissociating CaM with excess EGTA; a similar profile was observed for the wt nNOS (Figure 4). These FMN fluorescent response results imply that CaM binds to pSer1412 nNOS and induces protein conformational changes necessary for NO production.

The FMN – heme interdomain electron transfer kinetics of pSer1412 nNOS.

It is of current interest to study the discrete FMN – heme IET process [35, 36], which is catalytically essential in the delivery of electrons required for O_2 activation in the heme domain and the subsequent NO synthesis [2]. We have determined the FMN – heme IET kinetics in wt, pSer 1412 and S1412E rat nNOS proteins at 460 nm using laser flash photolysis (Figure 5), and the observed IET rates are listed in Table 1. It is of note that the FMN – heme IET is an equilibrium process: $\text{FMNH}^{\bullet} + \text{Fe(II)} \rightleftharpoons \text{FMN}_{\text{hq}} + \text{Fe(III)}$. The IET kinetics is thus measured in both directions, and the CO photolysis approach follows the IET process in the reverse direction of the NOS enzymatic turnover [3]. Because the observed IET rate is the sum of forward and reverse rate constants, the measurement of the IET in the reverse direction should not matter, though.

Importantly, the obtained rate constant is independent of the A460 signal amplitude (Figure S6), *i.e.*, concentration of the reduced protein, confirming an intraprotein process [2]. The IET rate constant of wt rat nNOS is also consistent with that of wt nNOS overexpressed in BL21(DE3) cells (Table 1) [16]. Note that the rate of the FMN – heme IET in pSer1412 nNOS is considerably lower than that of wt nNOS, while the S1142E mutant possesses a similar electron transfer rate to that of wt protein (Table 1). The obtained FMN – heme IET rates clearly demonstrate that (i) the protein is functional; and (ii) the pSer-incorporation is significant (otherwise the obtained IET rate of the pSer1412 nNOS protein should be the same as that of the wt protein, or we would observe a biphasic decay due to the two nNOS forms). A key finding here is that the FMN – heme IET rate in pSer1412 nNOS was reduced by ~ 33 %, which is a major reason to adopt our new protocol to study the effect of the Ser residue phosphorylation in NOS. These enzymatic activities and IET kinetic results thus demonstrate that it is necessary to utilize pSer-incorporated NOS, a true phosphorylated NOS form, in future biochemical and mechanistic studies.

Discussions

The observed FMN – heme IET rate is significantly reduced in pSer1412 nNOS relative to wt, while the rate of NO synthesis is not reduced upon phosphorylation of Ser1412 (Table 1). This contrasts with the observation that the S1412E phosphomimetic mutant shows a measurable change in NO synthesis rate, while the FMN – heme IET rate essentially remains unchanged (Table 1). Thus, there is an apparent absence of a link between the rate of FMN – heme IET and that of NO synthesis. In the literature, the FMN – heme IET step has very often been stated as the rate-limiting step in NOS catalysis [37], mainly because the IET step is the slowest among the interdomain electron transfer processes in NOS turnover [16]. However, our work shows that such view is overly simplistic and in fact incorrect. Our results show that the FMN – heme IET rate is clearly fast enough to not be rate-limiting in

NO synthesis by nNOS (Table 1) and the reduced IET rate (27 s^{-1}) in pSer1412 nNOS still results in a turnover rate of 1.14 s^{-1} . Moreover, the NO production activity is determined by the interplay of rates of the FMN – heme IET and two other heme related processes (NO dissociation from the ferric–NO complex and oxidation of the ferrous–NO complex) due to the role of NO as an intrinsic regulator [38].

While the ~ 33 % reduction in IET rate supports a native-like phosphorylation event, it is unclear what the functional consequence would be for phosphorylation at this site. Further functional evidence for a loss of IET is desirable. A critical aspect of NOS function is the generation of superoxide ($\text{O}_2^{\bullet-}$) radical, in which the heme reduction is a prerequisite for O_2 binding to the ferrous heme center and subsequent potential $\text{O}_2^{\bullet-}$ generation. Importantly, a growing body of evidence indicates that the phosphorylation affects nNOS coupling, the degree to which the catalytic cycle is coupled to NO production rather than reactive oxygen species including $\text{O}_2^{\bullet-}$. In fact, studies have shown a small but significant increase in $\text{O}_2^{\bullet-}$ production with insulin in neuronal cells when nNOS is phosphorylated [39]; phospho-nNOS at Ser1412 significantly increased between 1 h and 6 h after reperfusion in the hippocampus [40], indicating that nNOS phosphorylation at Ser1412 after cerebral ischemia could be a significant contributor to injury. Interestingly, phosphorylation of purified eNOS has also been shown to regulate $\text{O}_2^{\bullet-}$ generation from the enzyme [6]. However, due to lack of functional purified phospho-NOS proteins, the underlying mechanisms remain unclear. There is an urgent need to advance our understanding of the NOS phosphorylation-dependent NO/ $\text{O}_2^{\bullet-}$ redox signaling and its physiological/pathophysiological outcomes in neuronal and endothelial systems. It is therefore important to further determine if (and how) pSer1412 nNOS differs from the wt protein in $\text{O}_2^{\bullet-}$ production under substrate-depleted and abundant conditions. It will also be interesting to examine the effects of other phosphorylations and multi-phosphorylations on NOS function. These studies are underway and will provide further insight into the coupling of phosphorylation and function in this important enzyme.

An interesting question relates to why is the FMN – heme IET in pSer1412 nNOS protein is slower than wt. The observed IET rate is significantly affected by the conformational dynamics that determine the formation and dissociation of the docking complex between the FMN and heme domains [41, 42], yet much remains unknown regarding the mechanistic roles of NOS phosphorylations in the conformational transitions tied to discrete electron transfer steps. S1412 is located at the CT of rat nNOS. Due to the distant location of CT relative to the FMN domain, it is unlikely that phosphorylation of the S1412 residue would directly affect the docking geometry of the FMN-heme complex. Interestingly, a hydrogen-deuterium exchange mass spectrometry study recently showed that a phosphomimetic S→D mutation at the nNOS 1412 site modulates nNOS holoenzyme activity via long-range allostery [43]. Analyses of kinetics results for an eNOS phosphomimetic mutant at the equivalent site indicated that the eNOS phosphorylation may alter the conformational equilibrium within the reductase domain [7]. We thus suggest that the slower IET in pSer1412 nNOS may be more likely caused by changes in conformational dynamics, which include conformational change rates and docking equilibria related to the formation of the FMN-heme docking complex. The mechanism underlying the slower FMN – heme IET merits further investigation. We note that that detailed spectroscopic and fast kinetics studies

require milligram quantities of active phosphorylated NOS proteins, which were not accessible until now with our newly established preparation protocol.

In summary, we have shown, for the first time, that encoding pSer incorporation allows for high-level production of active phosphorylated nNOS protein. The purified recombinant pSer1412 holo-nNOS incorporates cofactor properly and displays NO production activity consistent with results reported in the literature for wt nNOS. Interestingly, the FMN – heme IET rate in pSer1412 nNOS is markedly slower than that of wt, while the S1412E mutant possesses similar kinetics to that of wt. The successful incorporation and high yield of pSer1412 into rat nNOS and the significant change in the IET kinetics demonstrate a highly useful method for incorporating native-like phosphorylation sites and represents a substantial improvement to commonly used phosphomimetics that may not recapitulate the phospho-protein's conformational dynamics and activities. This new pSer incorporation system will be useful for robust preparation of large amounts of active phospho-nNOS that can be effectively utilized in mechanistic and structure/function studies of NOS regulation by phosphorylation. Further spectroscopic and rapid kinetics studies on the phosphorylated NOS proteins are underway and will provide much needed insights into coupling of phosphorylation and function in this important family of redox enzymes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgement.

We thank Dr. Charles Melancon for helpful discussions. This work was supported by the National Institutes of Health (Grant No. GM-081811 to CF, GM-057378 to MLK and GM-081568 to LR). Mass spectrometry data were acquired by the University of Arizona Analytical and Biological Mass Spectrometry Facility supported by NIH/NCI grant University of Arizona.

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Figure 1.
Phosphomimetic substitution: aspartic acid/glutamic acid compared to phosphoserine.

Figure 2.

(A) Anti-phospho-nNOS (Ser1412) immunoblot analyses of purified wild type (wt; lane 1) and pSer1412 (lane 2) holo-nNOS expressed in *E. coli* C321. A harboring SepOTSλ with wt or pSer1412 nNOS expression plasmid grown without or with added 5 mM O-phospho-L-serine, respectively. (B) SDS-PAGE analysis of isolated proteins: lane 1, markers; lanes 2 and 3, purified wt and pSer1412 nNOS proteins (indicated by an arrow), respectively, eluted from a 2'5'-ADP Sepharose column. (C) Tandem mass spectrum of pSer1412 nNOS confirming the site-specific incorporation of pSer at position 1412.

Figure 3.
Far UV CD spectra of wt (red) and pSer1412 (blue) nNOS proteins (average of 16 scans).
The spectra were recorded at room temperature with 0.1 μ M protein in phosphate buffer (10 mM, pH 7.4).

Figure 4.

CaM binding induced change in the FMN fluorescence of nNOS. The nNOS protein was diluted to 1 μM in 25 mM phosphate buffer (pH 7.4), containing 100 mM KCl, 0.6 mM EGTA, 1 mM DTT, and 4 μM CaM. CaM binding to nNOS protein was induced by adding 1 mM calcium at the time indicated by the arrow. After five minutes, CaM was dissociated from the nNOS protein by adding 3 mM EGTA, as indicated by the arrow.

Figure 5.

Transient traces at 460 nm obtained for the $[\text{Fe(II)-CO}][\text{FMNH}^*]$ form of pSer1412 nNOS holoprotein flashed by 446 nm laser. Solid lines correspond to the best single-exponential fit to the data: upon a laser excitation, the absorption at 460 nm decays below the pre-flash baseline, which is due to the FMN-heme IET resulting in Fe^{2+} depletion. The sample temperature was set at 21 °C. Anaerobic solutions contained 15 μM pSer1412 nNOS, ~ 20 μM 5-deazariboflavin and 5 mM fresh semicarbazide in a pH 7.6 buffer (40 mM bis-Tris propane, 400 mM NaCl, 2 mM L-Arg, 20 μM H_4B , 1 mM Ca^{2+} and 10 % glycerol).

Table 1.

Rates of the FMN – heme IET (kIET) and NO synthesis of rat nNOS proteins at 21°C

	wt ^a	pSer 1412 ^a	S1412E ^b	wt ^b
kIET (s ⁻¹)	40 ± 2	27 ± 2	42 ± 2	47 ± 4
NO synthesis rate (s ⁻¹)	1.21 ± 0.12	1.14 ± 0.06	0.83 ± 0.01	0.94 ± 0.03

^aThe protein was overexpressed in *E. coli* C321. A cells.^bThe protein was overexpressed in *E. coli* BL21(DE3) cells.

Table 2.Rates of NO synthesis and NADPH oxidation in the presence of 100 μ M L-Arg ^a

	NO synthesis (min ⁻¹)	NADPH oxidation (min ⁻¹)	NADPH/NO
wt nNOS	72.4 $\pm$ 6.9	188.3 $\pm$ 23.2	2.6 $\pm$ 0.4
pSer1412 nNOS	68.5 $\pm$ 3.5	156.2 $\pm$ 13.5	2.3 $\pm$ 0.3

^a Rates reported are the average of at least three assays.