The Proximal Hydrogen Bond Network Modulates Bacillus subtilis Nitric-oxide Synthase Electronic and Structural Properties

Bacterial nitric-oxide synthase (NOS)-like proteins are believed to be genuine NOSs. As for cytochromes P450 (CYPs), NOS-proximal ligand is a thiolate that exerts a push effect crucial for the process of dioxygen activation. Unlike CYPs, this catalytic electron donation seems controlled by a hydrogen bond (H-bond) interaction between the thiolate ligand and a vicinal tryptophan. Variations of the strength of this H-bond could provide a direct way to tune the stability along with the electronic and structural properties of NOS. We generated five different mutations of bsNOS Trp66, which can modulate this proximal H-bond. We investigated the effects of these mutations on different NOS complexes (FeIIICO, FeIIIICO, and FeIINO), using a combination of UV-visible absorption, EPR, FTIR, and resonance Raman spectroscopies. Our results indicate that (i) the proximal H-bond modulation can selectively decrease or increase the electron donating properties of the proximal thiolate, (ii) this modulation controls the σ-competition between distal and proximal ligands, (iii) this H-bond controls the stability of various NOS intermediates, and (iv) a fine tuning of the electron donation by the proximal ligand is required to allow the same time oxygen activation and to prevent uncoupling reactions.

Nitrogen monoxide (NO) is a well described radical molecule (1) that has been shown to exert major physiological functions in mammals, ranging from signaling processes to cytotoxic activities (2–5). It is exclusively synthesized by a family of enzymes named nitric-oxide synthases (NOSs)2 that have been cloned and characterized in the early 1990s (6–9). With the emergence of efficient DNA sequencing techniques, the accessibility of an increasing number of genomes led to an unprecedented quest for new NOSs in other organisms. Although no NOSs have been found so far in plants and yeasts (10, 11), random BLAST analyses of several bacterial genomes led to the discovery of new NOS-like proteins, mostly in Gram-positive bacteria (12). These proteins correspond to the partially truncated oxygenase domain of mammalian NOSs (mNOSs). Because of the dominant structure-function approach, the first works on bacterial NOS-like proteins (bacNOSs) aimed at highlighting the similarities between mammalian and bacterial NOSs. Indeed, the crystallographic structures of NOSs from Bacillus subtilis (bsNOS (13)), Staphylococcus aureus (saNOS (14)), or Geobacillus steaothermophilus (15) were perfectly superimposable to the three-dimensional structure of mNOSs with the exception of a portion of the N-terminal region involved in BH4 binding and in the formation of the zinc-tetra-thiolate complex (12). In addition, the first enzymological experiments suggested that bacNOSs, similarly to mNOSs, had the capacity to catalyze oxygen activation and to metabolize L-Arg, the natural substrate of mNOSs, into citrulline and NO (16, 17). However, despite such an assignment of bacNOSs as genuine NO synthases, their in vivo function has been poorly investigated and remains a matter of debate. The first investigations on the biological function of bacNOSs suggested a contribution to the biosynthesis of a phytotoxin (thaxtomin A) of Streptomyces turgidiscabies, presumably via the nitration of a Trp-like residue on a diketopiperazine precursor (18, 19). This metabolic role seemed to extend to the Deinococcus radio-durans NOS, which seemed able to associate with the trytophanyl-tRNA synthetase and to nitrate Trp amino acids (20, 21). Following different hypotheses, Nuñez and colleagues (22–24) proposed that bacNOSs could intervene in a series of functions related to host-pathogen interaction such as protection against oxidative stress (22), pathogen survival and virulence (23), or defense against antibiotics (24). Recently, Crane suggested that D. radiodurans NOS could intervene in the recovery processes of bacteria subject to a UV stress (25). Because NO production is primarily used by the host as a bactericidal weapon, we feel that NO is not the most appropriate molecule to elicit a concerted response against the host immune system. In fact, our recent data suggest that bsNOS is more likely to intervene in NO and peroxynitrite detoxification (26). This raises the following question about the in vivo enzymatic activity of bacNOSs. Are they genuine nitric-oxide synthases?

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Albane Brunel, Adjèle Wilson, Laura Henry, Pierre Dorlet, and Jérôme Santolini

From the Commissariat à l’Energie Atomique, iBiTec-S, SB2SM, F-91191 Gif-sur-Yvette, France and CNRS, Laboratoire Stress Oxydant et Détoxication, URA 2096, F-91191 Gif-sur-Yvette, France
Effect of Proximal Ligation on NOS Stability and Activity

In comparison with mNOSs, the catalytic mechanism of bacNOSs has not been intensely investigated, but the results obtained so far stress numerous differences between mNOSs and bacNOSs. On top of them, bacNOSs are lacking an effective electron donor. The absence of a dedicated reductase partner (27) should prevent an efficient first electron transfer, although flavodoxins have been reported to support bsNOS-mediated NO production (28). No strong evidence has been reported so far for the implication of a pterin in the second fast electron transfer. The natural pterin is presumably not synthesized by these bacteria, and although BH4 and tetrahydrofolate increase the rate of decay of the Fe\(^{II}\)O\(_2\) complex of several bacNOSs (16, 29, 30), no pterin radical has been identified in bacNOSs catalysis so far. Another problematic issue is the NO dissociation rate of the bsNOS Fe\(^{II}\)NO complex. Indeed, the Val → Ile substitution observed in the bacNOSs heme pocket seems to impede a fast NO release (30), which is mandatory for NO synth-

**SCHEME 1.** Crystallographic structure of the active site of bsNOS highlighting the interactions between the heme, the proximal thiolate, and tryptophan 66. This structure was generated from the crystallographic structure of native bsNOS (Protein Data Bank entry 1M7V) (13) by using the Swiss-Pdb viewer Deepview and PovRay software (both available on the World Wide Web). This image illustrates the H-bond between Trp\(^{66}\) and the thiolate ligand, as well as the π-stacking between the tryptophan and the porphyrin.

...workers (53), who showed that the suppression of the Trp-Cys H-bond in saNOS seemed to modify its proximal Fe–S bond.

We report here a comprehensive investigation of the modulation of the interaction between the proximal cysteine ligand and the vicinal Trp for bsNOS. We analyzed the effects of several mutations (W66A/L/F/Y/H) on the spectral fingerprints of bsNOS key species by using a combination of resonance Raman, ATR-FTIR, and EPR spectroscopies. Our results show that the modulation of this interaction not only modifies the electron donation of the thiolate ligand but also affects the electronic structure of the Fe\(^{II}\)NO complex. This seems to indicate that the reactivity of NOS major reactive intermediates (Fe\(^{III}\)O\(_2\) and Fe\(^{II}\)NO) is controlled by the same means (the proximal H-bond network) in bacterial and mammalian NOSs.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—All chemicals were purchased from Sigma-Aldrich. BH\(_4\) and \(N^\omega\)-hydroxy-L-arginine were purchased from Enzo Life Sciences (Enzo Life Sciences Inc., Farmingdale, NY). NO and CO gases were purchased from Messer France SA (Asnières, France). NO-saturated solutions were freshly prepared by flushing NO gas through a previously degassed 100 mM potassium phosphate (KP) buffer at pH 7.4.

**Molecular Biology**—The bsNOS gene was a kind gift of Dr. Dennis J. Stuehr (16).\(^3\) Wild-type bsNOS and mutants containing a Histag attached to their N terminus were overexpressed in Escherichia coli strain BL21 (DE3) using a pET15B expression vector as described (16). Trp\(^{66}\) mutations were added in the bsNOS gene using the QuikChange XL site-directed mutagenesis kit from Stratagene and synthetic mutagenic oligonucleotides. Oligonucleotides used to construct site-directed mutants in bsNOS were synthesized by Eurofins MWG. Silent mutations coding for the disappearance of the EcoRI (GAATTTC) restriction site were incorporated into the oligonucleotides to aid in screening. Mutations (boldface type), the EcoRI restriction site

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\(^3\) It should be noted that the bsNOS used in Stuehr’s laboratory (strain spizizenii W23) differs slightly from the one used in Crane’s laboratory (strain 168) by a small N-terminal extension, leading to a shift in the residue numbering.
Effect of Proximal Ligation on NOS Stability and Activity

Protein Expression and Purification—Wild-type and Trp66 mutant bsNOS were expressed in E. coli. 400-ml cultures of terrific broth containing 125 mg/liter ampicillin were initiated with 500 µl of stock glycerol bacterial culture and stirred at 250 rpm at 37 °C. At A600 = 0.8–1, starter cultures (400 ml) were used to inoculate 3.6 liter of the same medium. Protein expression was induced at A600 = 1 by adding 1 mM isopropyl-β-D-thiogalactoside, and the cultures were supplemented with 500 µM δ-aminolevulinic acid. After 12 h of growth at 20 °C, the cells were harvested by centrifugation at 6000 rpm for 20 min at 4 °C and resuspended in ice-cold lysis buffer (0.1 M Tris-HCl, pH 8–9, with 10% glycerol, 1 mM EDTA, and 0.25 M NaCl) containing 1 mg/ml lysozyme, 0.5 µg/ml each leupeptin and pepstatin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 50 units/ml DNase I (bovine pancreas type IV, Sigma) with or without 10 mM Arg (10 mM) and/or BH4 (100 µM). Cells were lysed by two cycles of French press at 700 p.s.i. The lysate was centrifuged at 16,000 rpm for 45 min at 4 °C. (NH4)2SO4 (50–55% w/v, final concentration) was added. After mixing for 30 min and centrifugation (30 min, 16,000 rpm at 4 °C), the pellet was resuspended in binding buffer (0.1 M Tris-HCl, pH 8–9, 10% glycerol, 0.25 M NaCl, 1 mM PMSF with or without 10 µM Hb and 5 mM l-arginine). The supernatant was loaded on a column of Ni-ProBond resin (Invitrogen) pretreated with binding buffer containing 200 mM imidazole. Column fractions were pooled and were concentrated using Centricon membrane concentrators with a 30 kDa cut-off (Millipore). For samples containing BH4, a final dilution/concentration cycle with freshly prepared BH4 buffer was performed just before the measurements.

Anerobic ferric NOS (FeIII) was first prepared by 100–200 cycles of alternate vacuum and argon refilling, directly in a quartz EPR tube (EPR and resonance Raman experiments) or in a quartz cuvette (ATR-FTIR and UV-visible spectrometry), both sealed with air-tight rubber septa. Ferrous samples (FeII) were reconditioned in a KPi (100 mM, pH 7.4) buffer in the presence of different combinations of Arg (10 mM) and/or BH4 (100 µM to 1 mM) by three successive cycles of dilution/centrifugation in the final buffer using MicroCon membrane concentrators with a 30 kDa cut-off (Millipore). For samples containing BH4, a final dilution/concentration cycle with freshly prepared BH4 buffer was performed just before the measurements.

Preparation of WT and Mutant bsNOS Complexes—Samples were reconditioned in a KPj (100 mM, pH 7.4) buffer in the presence of different combinations of Arg (10 mM) and/or BH4 (100 µM to 1 mM) by three successive cycles of dilution/centrifugation in the final buffer using MicroCon membrane concentrators with a 30 kDa cut-off (Millipore). For samples containing BH4, a final dilution/concentration cycle with freshly prepared BH4 buffer was performed just before the measurements.

for protein expression.

ATR-FTIR Spectroscopy—Room temperature FTIR spectra were recorded using a Bruker IFS 66 Fourier transform infrared spectrometer (Bruker Optik GmbH, Ettlingen, Germany) coupled to a single reflection micro-ATR prism from Pike Technologies (Madison, WI). 30 µl of 500 µM bsNOS FeII(CO) sample was prepared in a small quartz cuvette as described above. 10 µl were deposited using a gas-tight syringe on the ZnSe crystal surface of the ATR unit. The crystal was sealed within a gas-tight in-house built chamber, which permitted the control of the atmosphere above the sample. Twenty-fold 250 co-added interferograms were averaged for each FTIR measurement. A water vapor spectrum was used for background correction. Base-line correction was achieved using the GRAMS 32 soft-
Properties, we mutated the bsNOS Trp66 residue into five different amino acids (see "Experimental Procedures"): a phenylalanine (W66F) and a tyrosine (W66Y) to maintain the aromatic ring but remove the H-bond; a histidine (W66H) that is believed to maintain and even strengthen this H-bond (52); a leucine (W66L), with the main difference observed in the absence or presence of l-Arg. In both cases, the dominant features correspond to a signal from the high spin pentacoordinated (HS-5c) Fe$^{III}$ heme moiety. This signal was simulated, taking into account the electronic Zeeman interaction (taken as isotropic) and the zero-field splitting interaction characterized by the parameters $D$ and $E$. Because the zero-field splitting interaction is largely dominant over the Zeeman interaction, the important parameter obtained from the simulation is the ratio $E/D$ that is listed in Table 1. The simulated spectra are displayed in Fig. 1 together with the experimental data. In the absence of substrate, the $E/D$ value increases in the order WT $<$ W66H $<$ W66L, with the main difference observed between the two groups WT/W66H and W66Y/F/L. The addition of l-Arg to the proteins greatly increases the structural homogeneity of the samples as indicated by the EPR spectra, which now display only a HS-5c Fe$^{III}$ signal with features narrower than those observed in the absence of substrate. The rhombicity of the HS signal varies continuously between the different bsNOS with the order W66L $<$ WT $<$ W66Y $<$ W66F $<$ W66H.

In the absence of substrate, all bsNOSs exhibit a fraction of low spin hexacoordinated (LS-6c) species. This is particularly true in the case of W66Y and W66F, whereas the proportion of LS-6c heme is much lower in the case of WT, W66H, and W66L. The LS-6c EPR spectra are shown in Fig. 2 for WT and W66H/Y/F along with the simulated spectra, and the parameters used for the simulations are listed in Table 2. Also for those low spin species, an effect of the mutation is observed on the EPR spectra, with the W66H mutant being similar to the WT, whereas the W66Y and W66F exhibit lower g-anisotropy and isotropic g. The Trp$^{66}$ mutation affects the rhombicity of both LS-6c species (in the absence of l-Arg) and HS-5c species (in the absence or presence of l-Arg).

These EPR results are in agreement with the UV-visible data recorded on wild-type and mutated bsNOS samples that show a predominant HS-5c for all proteins in the presence of substrate and cofactor and the presence of a fraction of LS-6c species in the absence of both cofactor and substrate (see Table 3 and supplemental Fig. S1).

**UV-visible and Resonance Raman Investigation of the Fe$^{III}$CO Complexes of bsNOS Proteins**—We recorded the UV-visible spectrum of wild-type and mutated bsNOS in the presence of substrate and cofactor.
The C–O bond is stronger for the W66H FeIICO complex than for WT and weaker for the W66F/Y mutants. The concomitant increase of the Fe–C bond is the strongest for W66H FeIICO complex and then weakens for WT and weaker for the W66F/Y mutants. The binding of L-Arg modifies the equilibrium between the P420 and P450 forms but also the wavelength of the Soret band, confirming that all mutants efficiently bind their natural substrate (Table 3 and supplemental Fig. S2). We also analyzed the binding of imidazole, a distal ligand of ferric NOS. We found that imidazole binds and converts bsNOS WT and W66H/F/Y mutants into a LS-6c complex with a Soret maximum around 426 nm (data not shown), as observed for other bacterial and mammalian NOSs (64–67). However, the dissociation constant was found to vary as a function of the Trp66 mutation. The affinity of imidazole was the highest for W66H ($K_d = 0.4$ mM), followed by WT ($K_d = 0.95$ mM), W66F ($K_d = 3$ mM), and finally W66Y ($K_d = 6.5$ mM).

We recorded the resonance Raman spectra of the Fe$^{III}$CO complex of the W66F, W66Y, and W66H mutants in the presence of both substrate and cofactor. They all indicate the major presence of an LS-6c Fe$^{III}$CO complex, with some minor photodissociation contribution (data not shown) such as what we observed for the wild-type bsNOS (43). We analyzed the 450–600 cm$^{-1}$ spectral region (Fig. 3B). Spectra were deconvolved by a multi-Lorentzian function as described under “Experimental Procedures.” The peak that corresponds to the $\delta_{Fe-CO}$ bending mode (40, 43, 45) was observed around 567 cm$^{-1}$ for wild-type and mutated NOSs (Table 3), indicating the absence of significant alteration of the Fe$^{III}$CO complex geometry. Whereas wild-type Fe$^{III}$CO complex exhibits a $\nu_{Fe-CO}$ frequency around 501 cm$^{-1}$ (43), this frequency decreases to 500 cm$^{-1}$ for W66F mutant but increases up to 504.5 cm$^{-1}$ for W66H bsNOS (Table 3). These data indicate that the Fe–C bond is the strongest for W66H Fe$^{III}$CO complex and then weakens for WT bsNOS and for W66F/Y mutants. Fig. 3A exhibits the ATR-FTIR spectra of the same complexes. We noticed a similar modification of the frequency of the $\nu_{Fe-CO}$ stretching mode that was observed around 1920 cm$^{-1}$ for W66H mutant, 1917 cm$^{-1}$ for WT bsNOS, and 1913 cm$^{-1}$ for W66F/Y mutants (Table 3). The C–O bond is stronger for the W66H Fe$^{III}$CO complex than for WT and weaker for the W66F/Y mutants. The concomitant decrease (respectively increase) of the $\nu_{Fe-CO}$ and $\nu_{CO}$ stretching frequencies reflects an increase (respectively a decrease) of absorption maximum of the Soret band varies with the mutant (Table 3). Wild-type and mutant complexes were obtained in saturating conditions of L-Arg and H$_4$B. The top part of the table shows Soret wavelength (in nm) for different NOS complexes. The bottom part shows wave numbers (in cm$^{-1}$) of different stretches of Fe$^{III}$CO complexes: 450/420, hexa- and pentacoordinated Fe$^{III}$CO complexes, respectively. Values in parentheses correspond to full width at half-maximum values in cm$^{-1}$.

### TABLE 3

**Comparison of spectral characteristics of various complexes of wild-type bsNOS and Trp$^{66}$ mutants**

| Compound | WT | W66H | W66F | W66Y |
|----------|----|------|------|------|
| Fe$^{III}$ | 450 | 446.5 | 441.5 | 449 | 448.5 |
| Fe$^{III}$NO | 438 | 434.5 | 438 | 435 | 419.5 |
| Fe$^{III}$NO | 439.5 | 433 | 435.5 | 436 | 436 |
| $\nu_{Fe-CO}$ | 567 | 567 | 566 | 567 |
| $\delta_{Fe-CO}$ | 1917 (9) | 1920 (11) | 1913 (10) | 1913 (11) |

**TABLE 2**

**Simulation parameters for the EPR spectra of the LS Fe$^{III}$ heme for wild-type and mutated bsNOS**

| $g_1$ (H strain) | $g_2$ (H strain) | $g_3$ (H strain) | $g_{iso}$ | $\Delta g (g_{iso} - g_1)$ |
|------------------|------------------|------------------|-----------|--------------------------|
| WT | 2.447 (500) | 2.292 (0) | 1.900 (200) | 2.213 | 0.547 |
| W66H | 2.450 (450) | 2.288 (0) | 1.900 (157) | 2.213 | 0.550 |
| W66Y | 2.409 (360) | 2.274 (0) | 1.928 (103) | 2.204 | 0.481 |
| W66F | 2.392 (260) | 2.267 (0) | 1.931 (77) | 2.197 | 0.461 |

Simulation parameters for the EPR spectra of the LS Fe$^{III}$ heme for wild-type and mutated bsNOS. Shown are the $g_1$ strain values in MHz (full width at half-height line width describing broadening due to unresolved hyperfine couplings). $g_{iso}$ is the average of the three principal values $g_1$, $g_2$, and $g_3$.

Both L-Arg and BH$_4$. Characteristic Soret band absorption maxima are listed in Table 3 (see also supplemental Fig. S2). WT and W66H Fe$^{III}$CO complexes are mostly hexacoordinated. W66L, Fe$^{III}$CO complex exhibits a Soret band at 420 nm characteristic of a weakening of the proximal ligation (58, 61), supporting a weakening of the proximal ligation (58, 61), suggesting again an opposite modification of the electron donation of the proximal ligand for the W66H and the W66F/Y mutants. The binding of L-Arg modifies the equilibrium between the P420 and P450 forms but also the wavelength of the Soret band, confirming that all mutants efficiently bind their natural substrate (Table 3 and supplemental Fig. S2). We also analyzed the binding of imidazole, a distal ligand of ferric NOS. We found that imidazole binds and converts bsNOS WT and W66H/F/Y mutants into a LS-6c complex with a Soret maximum around 426 nm (data not shown), as observed for other bacterial and mammalian NOSs (64–67). However, the dissociation constant was found to vary as a function of the Trp$^{66}$ mutation. The affinity of imidazole was the highest for W66H ($K_d = 0.4$ mM), followed by WT ($K_d = 0.95$ mM), W66F ($K_d = 3$ mM), and finally W66Y ($K_d = 6.5$ mM).

The instability of the proximal bond of the FeIICO complex significantly increases for all proteins in the absence of L-Arg and BH$_4$. The absorbance maximum of the Soret band varies with the mutant (Table 3). Wild-type bsNOS exhibits a Soret wavelength around 446.5 nm. This band is blue-shifted for W66H (441.5 nm) and red-shifted for W66F and W66Y mutants (449 and 448.5 nm, respectively), suggesting again an opposite modification of the electron donation of the proximal ligand for the W66H and W66F/Y mutants. The binding of L-Arg modifies the equilibrium between the P420 and P450 forms but also the wavelength of the Soret band, confirming that all mutants efficiently bind their natural substrate (Table 3 and supplemental Fig. S2).
the α-competition between the distal and proximal ligands of the W66F/Y (respectively W66H) mutants (43, 45, 55).

Characterization of Heme–NO Complexes of Wild-type and Mutant bsNOS—The Fe^{II}NO complexes of WT and W66H/F/Y mutants were found to be relatively stable both in the absence and presence of substrate and cofactor (see supplemental Fig. S3 for the UV-visible spectra). The mutation mostly affects the values of the Soret band maximum that varies as follows: 433 (W66H) and 440 nm (WT; Table 3). The Fe^{II}NO complexes of the bsNOS mutants also display spectral fingerprints similar to those of WT bsNOS in the presence of both l-Arg and BH$_4$. Only the W66Y Fe^{II}NO spectrum was found to exhibit a shoulder around 417 nm that is reminiscent of what was observed for the nNOS W409F/Y mutant (60, 68). Here again, the wavelength of the Soret band maximum was observed between 433 (W66H) and 440 nm (WT; Table 3).

The Fe^{II}NO complexes of bsNOS proteins were also analyzed by EPR spectroscopy (Fig. 4). In the absence of l-Arg and BH$_4$, the observed spectra were dominated by the well known rhombic powder pattern with three different g-values and resolved hyperfine coupling with the nitrogen nucleus of the NO ligand (Fig. 4A). The EPR spectra were simulated for all hexacoordinated species (WT and W66H/F/Y) as well as for the pentacoordinated complex of W66L. Taking into account the Zeeman interaction and the hyperfine coupling to the nitrogen nucleus of the NO ligand. Simulation parameters are reported in Table 4.

The EPR spectra were simulated for all hexacoordinated species (WT and W66H/F/Y) as well as for the pentacoordinated complex of W66L. Taking into account the Zeeman interaction and the hyperfine coupling to the nitrogen nucleus of the NO ligand. Simulation parameters are reported in Table 4. With respect to the hexacoordinated species (WT and W66H/F/Y), the total g-anisotropy (Δg) of the Fe^{II}NO signal increased with the order W66H < WT < W66F < W66Y, whereas the isotropic g-value (g$_{iso}$) decreased in the same order (Table 4). The isotropic hyperfine coupling of the nitrogen nucleus of the NO molecule also decreased in the same order, indicating that the spin density on the NO nitrogen is the greatest on W66H and decreases slightly for WT > W66F > W66Y. This indicates that Trp$_{66}$ mutations modify the electronic properties of Fe^{II}NO complexes.

DISCUSSION

Trp$_{66}$ of bsNOS is engaged in an H-bond with the heme proximal ligand that is believed to tune the properties of the NOS Fe–S bond (53). We present here for the first time a comparative analysis of five different mutants of this residue that differentially affect the environment of the proximal ligand: (i) the F and Y mutants, already described for nNOS (70) and recently for eNOS and saNOS (53), correspond to a suppression of the H-bond; (ii) the H mutant, described for iNOS (52), is supposed to induce a strengthening of the H-bond; (iii) finally, the A and L mutants, as yet undescribed, should suppress both H-bond and π-stacking interactions. A comparative analysis of
the effects of these different mutations on bsNOS structural and electronic properties should allow a better understanding of the way this H-bond modulates NOS structure and function.

Control of the \( \sigma \)-Competition between NOS-distal and -proximal Ligands—The Fe(II)CO complex is commonly used as a probe to analyze the influence of heme environment on Fe(III)O2 structure and reactivity (55, 71, 72). Vibrational spectroscopies, such as resonance Raman and FTIR spectroscopies, allow the characterization of \( \nu_{\text{Fe-CO}} \) and \( \nu_{\text{C-O}} \) stretching modes. The frequencies of these modes are sensitive to the electronic properties of the proximal ligand and to polar and/or steric interactions between CO and the heme distal pocket (71, 72). Modifications of the electrostatic/polar distal environment of the CO ligand result in changes in the back donation from the iron d\( \pi^* \) orbital to the empty \( \pi^* \) orbital of CO. This leads to the well known inverse correlation between the frequencies of the \( \nu_{\text{Fe-CO}} \) and \( \nu_{\text{C-O}} \) (45). Additionally, there is a competition between the proximal and distal ligands for \( \sigma \)-bonding to the \( d_z^2 \) orbital of the heme iron. Variations in the electronic properties of the proximal ligand will impact this competition and shift the \( \nu_{\text{Fe-CO}}/\nu_{\text{C-O}} \) correlation curve. Strong electron-donating ligands (such as the thiolate of NOS and cytochromes P450) will shift correlation lines toward low \( \nu_{\text{Fe-CO}} \) and \( \nu_{\text{C-O}} \) frequencies, whereas weak proximal ligand (in the case of globins or some peroxidases) will lead to higher \( \nu_{\text{Fe-CO}} \) and \( \nu_{\text{C-O}} \) frequencies.

Our results suggest that the mutations do not affect Fe(II)CO geometry (no change in the bending modes; Table 3). However, they show an increase in the \( \nu_{\text{Fe-CO}} \) and \( \nu_{\text{C-O}} \) frequencies for the W66H mutant, whereas the W66F and W66Y mutants are characterized by weaker Fe–CO and C–O bonds (Table 3). Plotted in the \( \nu_{\text{Fe-CO}}/\nu_{\text{C-O}} \) correlation graph (data not shown), these data show a change in the offset of the correlation line, univocally linked to the variation of the proximal ligand strength. This indicates that the mutation directly modifies the electron donating ability of the proximal thiolate; W66F and W66Y are characterized by stronger electron donation (in agreement with the removal of the H-bond), whereas W66H is characterized by weaker electron donation (in agreement with a strengthening of the H-bond).

In this report, using the inverse \( \nu_{\text{Fe-CO}}/\nu_{\text{C-O}} \) Correlation, we are able for the first time to probe within a single NOS protein both the increase and the decrease of this \( \sigma \)-competition and to assign these variations to the modifications of the H-bonding interaction in which the proximal ligand is engaged.

Role of Tryptophan-Thiolate H-bond in bsNOS Stability—This variation in \( \sigma \)-competition has strong effects on the properties of bsNOS complexes, as reflected by the effects of the mutation on Fe(II)NO and Fe(II)CO stability (Fig. 4 and supplemental Figs. S2 and S3). We observed a greater proportion of pentacoordinated forms of these complexes for W66F and W66Y bsNOS (due to a stronger \( \sigma \)-competition), whereas W66H hexacoordinated complex (with a weaker proximal ligand) seems as stable as (if not more stable than) WT complexes. The same trend is observed in the dissociation constants of the Fe(III)-Im complex. The affinity of imidazole for bsNOS heme is the greatest for W66H (weak \( \sigma \)-competition), decreases for WT, and decreases even more for W66F and W66Y (strong \( \sigma \)-competition). However, we do not observe a better stabilization of Fe(II)CO and Fe(II)NO species for W66Y; no H-bond with the tyrosine proton, such as the one observed for eNOS (53), can be deduced from our data. Our work clearly indicates that the tryptophan-thiolate H-bond interaction controls the stability of bsNOS Fe(II)-XO complexes, such as Fe(II)NO, Fe(II)CO, and most probably Fe(II)O2.

Additionally, our study reveals that other structural features contribute to bsNOS stability. Indeed, upon binding of a distal ligand, such as CO or NO, the W66L complex is quickly and fully converted into pentacoordinated species, indicating the loss of the proximal thiolate ligand. Compared with W66F and W66Y, this further decrease in stability might arise from the loss of specific interactions that stabilize NOS heme, such as the \( \pi \)-stacking between the tryptophan indole ring and the porphyrin cofactor (Scheme 1). Furthermore, the W66A mutant exhibits a critical instability of the protein, as illustrated by the severe difficulties of purification. Accordingly, the EPR spectrum of the W66A ferric enzyme (data not shown) suggests a complete unfolding of the protein and a partial loss of the heme.

Effect of Trp66 Mutations on the Electronic Structures of Fe(II)-XO Complexes—The variations in the electron-donating properties of the proximal thiolate seem to also exert an impact on the electronic structure of bsNOS complexes. For example, the Soret maximum of the Fe(II)CO spectra is red-shifted for W66F and W66Y mutants (a stronger electron-donating ligand) and blue-shifted for W66H bsNOS (weaker proximal ligand). These variations are also reflected by the shifts of the Soret maxima of Fe(II)NO and Fe(II)NO complexes (Table 3) and to a lesser extent by the modification of the low spin/high spin equilibrium. The variations of bsNOS electronic properties can also be appreciated by EPR spectroscopy. The g-anisotropy of LS-6c Fe(III) species, the minor fraction of native bsNOS, is greater for W66H/WT than for W66F/W66Y (Fig. 2 and Table 2). A similar distinction between W66F/W66Y on one hand and W66H/WT on the other hand is observed for the zero-field splitting rhombicity of the prevalent Fe(III) HS-5c species. Indeed, the \( E/D \) value is clearly greater for the W66F/W66Y

### Table 4

| **Mutation** | **\( g_{\text{Fe-CO}} \) (g strain)** | **\( g_{\text{C-O}} \) (g strain)** | **\( g_{\text{Fe-CO}} \) (g strain)** | **\( \Delta g_{\text{Fe-CO}} \) (g strain)** | **\( A_1 \) (MHz)** | **\( A_2 \) (MHz)** | **\( A_3 \) (MHz)** | **\( A_{\text{iso}} \) (MHz)** |
|--------------|-----------------------------------|-----------------------------------|-----------------------------------|------------------------------------|------------------|------------------|------------------|------------------|
| WT           | 2.0816 (0.0075)                   | 2.0046 (0.0010)                   | 1.9671 (0.0054)                   | 2.0178                            | 0.1145           | 28.8             | 59.9             | 32.5             | 40.1             |
| W66F         | 2.0808 (0.0075)                   | 2.0046 (0.0010)                   | 1.9737 (0.0064)                   | 2.0197                            | 0.1071           | 32.0             | 59.0             | 37.0             | 42.7             |
| W66Y         | 2.0771 (0.0062)                   | 2.0043 (0.0010)                   | 1.9615 (0.0047)                   | 2.0143                            | 0.1156           | 26.0             | 57.0             | 31.0             | 38.0             |
| W66F         | 2.0760 (0.0050)                   | 2.0037 (0.0010)                   | 1.9610 (0.0045)                   | 2.0146                            | 0.1150           | 27.6             | 60.0             | 31.1             | 39.6             |
| W66L         | 2.1040 (0.010)                   | 2.0094 (0.0030)                   | 2.0094 (0.0030)                   | 2.0409                            | 0.1150           | 42.0             | 50.5             | 50.5             | 47.7             |
Effect of Proximal Ligation on NOS Stability and Activity

mutants than for W66H and WT (Fig. 1 and Table 1). The same variations are observed for hexacoordinated FeIII NO complex. The anisotropy is greater for W66F/W66Y than for W66H and WT bsNOS (Table 4).

The effects of the variation of the proximal bond on the detailed electronic structure of FeIII NO complexes are more difficult to analyze. However, the comparison of the EPR fingerprints for this series of mutants indicates a significant contribution of the proximal H-bond in the distribution of the electronic density on the FeIII NO moiety. The spin density on the NO nitrogen seems maximal for W66H and decreases as the hydrogen bond strength on the proximal thiolate ligand is decreased (Table 4). These results show that changes in the electron-donating properties of the proximal ligand drastically modify the electronic structure of FeIII and FeIII NO complexes.

Influence of the Proximal H-bond on FeII-XO Reactivity—The variations of NOS electronic structure induced by the changes in the proximal H-bond interactions are believed to modify its catalytic activity. Indeed, the rates of FeIIIO2 autoxidation and FeIIIO oxidation increase for W409F/Y nNOS (31, 70). Reciprocally, the strengthening of this H-bond in the case of W188H nNOS induces a decrease of FeIIIO2 autoxidation and activation rates that eventually allow the observation of new reaction intermediates (52).

All of these results suggest that bacterial NOSs use the same regulation feature, the “push effect” (73), to finely tune its oxidative chemistry. They also underline the necessity to precisely delimit the proximal ligand electron donation. A too strong electron donation (e.g., for Phe/Tyr mutants) exacerbates the instability of NOS reaction intermediates and mostly leads to uncoupling reactions due to a stronger electron density on the heme species production. A weak electron donation (e.g., for the His mutant) leads to an insufficient reactivity of iron-oxo complexes (74).

The functional analysis of our panel of mutants by the Griess assay (see supplemental Fig. S4 and Table S1) confirms this model. On one hand, the W66H mutant exhibits an extremely weak nitrite synthase activity (6 × 10−4 s−1), which corresponds to around 5% of WT standard activity. On the other hand, W66F activity remains comparable with that of WT bsNOS (around 50%). This difference could arise from uncoupling reactions due to a stronger electron density on the heme intermediates.

CONCLUSION

We present here a comprehensive analysis of the role of the H-bond in the control of NOS structural and electronic properties. We analyzed the effects of both suppression (W66F/Y) and strengthening (W66H) of this H-bond on FeIII, FeII CO, and FeIII NO spectroscopic fingerprints.

We evidenced for the first time a tight correlation between the proximal H-bond network, the electron-donating properties of the proximal ligand, and the stability and reactivity of NOS. Our data indicate that the removal of the H-bond (W66F/Y) increases the electron donation properties of the proximal ligand, which in turn induces an increased α-competition on the iron orbitals. This competition leads to the destabilization of FeIII-XO complexes, such as FeIII NO, FeII CO, and putatively FeIIIO2. This destabilization is reverted when the Phe/Tyr residues are replaced by a histidine that restores (and even strengthens) the proximal H-bond and thus diminishes the α-competition. These results confirm and complete previous information obtained for the Phe/Tyr mutants of saNOS (Trp66), eNOS (Trp180) (53), and nNOS (Trp409) (60, 68) and highlight the crucial role of this proximal H-bond in the stability of NOS reactive intermediates.

Additionally, our results suggest that bacterial NOSs are genuine oxidoreductases that seem to utilize the electron donation from the thiolate to activate distal ligands, such as O2 and NO, and to use the same regulation feature, namely the proximal H-bond to tune and control their catalytic activity. This pattern could also intervene in the interaction between NOS and other reactive ligands, such as peroxynitrite. A comparative analysis of the role of this H-bond in the mechanisms of dioxygen activation, of FeIIIO oxidation, or of peroxynitrite activation could help understand the biological specificities of mammalian and bacterial NOSs.

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