Supplemental data for

INFLUENCE OF HEME-THIOLATE IN SHAPING THE CATALYTIC PROPERTIES OF A BACTERIAL NITRIC OXIDE SYNTHASE*

Luciana Hannibal, Ramasamy Somasundaram, Jesús Tejero, Adjele Wilson and Dennis J. Stuehr

From the Department of Pathobiology, Lerner Research Institute,

Cleveland Clinic, Cleveland, Ohio 44195 and Laboratoire stress oxidant et detoxication (LSOD), CEA-IBITEC-S, Saclay, France.

Running title: Role of a proximal Trp residue in catalysis by bsNOS

Address correspondence to: Dennis J. Stuehr, Department of Pathobiology (NC-22), Lerner Research Institute, The Cleveland Clinic Foundation, 9500 Euclid Ave., Cleveland, Ohio 44195.

Phone: 216-445-6950; Fax: 216-636-0104; E-mail: stuehrd@ccf.org.

Figure S1. Purity and oligomeric state of bsNOS wt and W66 mutants.

Figure S2. Spectrophotometric titrations of L-Arg (upper panels) and NOHA (lower panels) and determination of the binding affinity constants, K_d.

Figure S3. Single turnover reactions with L-Arg and H_4T of bsNOS wild type (A-C) and mutant proteins W66H (D-F) and W66F (G-I).

Figure S4. Single turnover reactions with NOHA and H_2T for bsNOS wild type, and mutant proteins W66H and W66F.

Figure S5. HPLC chromatograms for the formation of [^{14}C]-NOHA from [^{14}C]-L-Arg under single turnover conditions, at infinite time (10 min).

Table S1. UV-vis absorption maxima of bsNOS and mutants W66H and W66F reconstituted with H_4F/DTT and L-Arg or imidazole.

Table S2. Observed rate constants for the reactions of bsNOS, W66H and W66F in the presence of H_2B with L-Arg as substrate.

Table S3. Observed rate constants for the reactions of bsNOS, W66H and W66F in the presence of H_2T, with L-Arg or NOHA as substrates.

Table S4. Rates of Fe(II)O_2 complex conversion to Fe(III) of selected hemeproteins.

Table S5. Maximum yields for the conversions of L-Arg to NOHA and NOHA to citrulline at infinite time by wild type and mutant *B. subtilis* NOSs in reactions performed with H_4T.

Table S6. Oxidation rates of Fe(III)-NO complexes of selected hemeproteins.

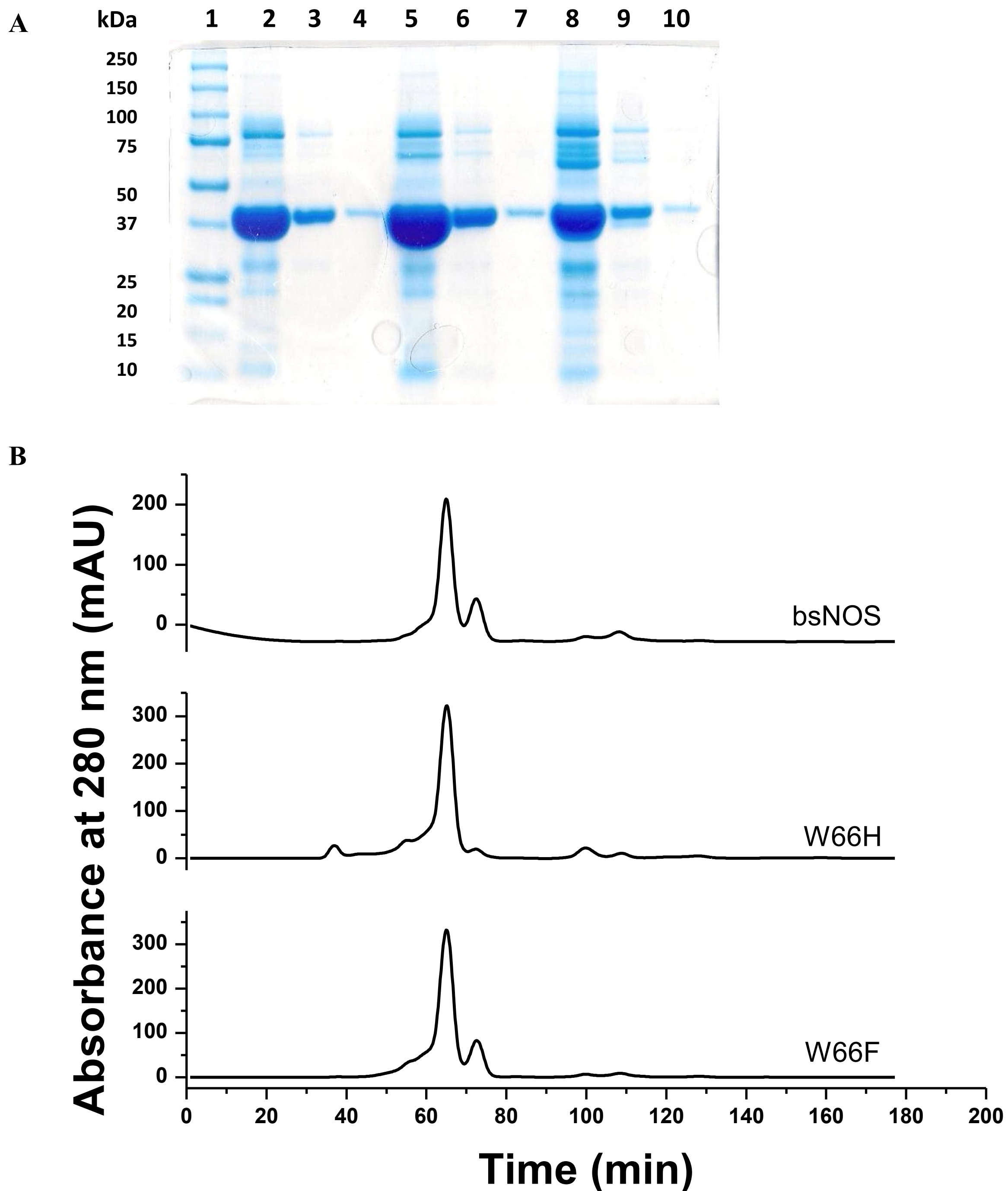

Figure S1. Purity and oligomeric state of bsNOS wt and W66 mutants. **A.** SDS-PAGE (10%) of bsNOS (lanes 2-4), W66F(lanes 5-7) and W66H (lanes 8-10). Serial dilutions (1:10 and 1:100) are shown. **B.** Size exclusion chromatography of bsNOS and W66 mutants reconstituted with L-Arg and H_4T. Each protein (~ 150 μM) was incubated for with 1 mM L-Arg, 400 mM H_4T and 1.2 mM DTT for 10 min. The samples were run on a Superdex 200 column pre-equilibrated with buffer (40 mM EPPS, pH 7.6, 150 mM NaCl) containing 100 μM L-Arg, 40 μM H_4T and 120 μM DTT. Under these conditions (flow rate 0.2 mL/min), dimeric and monomeric NOS eluted at 65 and 73 min, respectively.

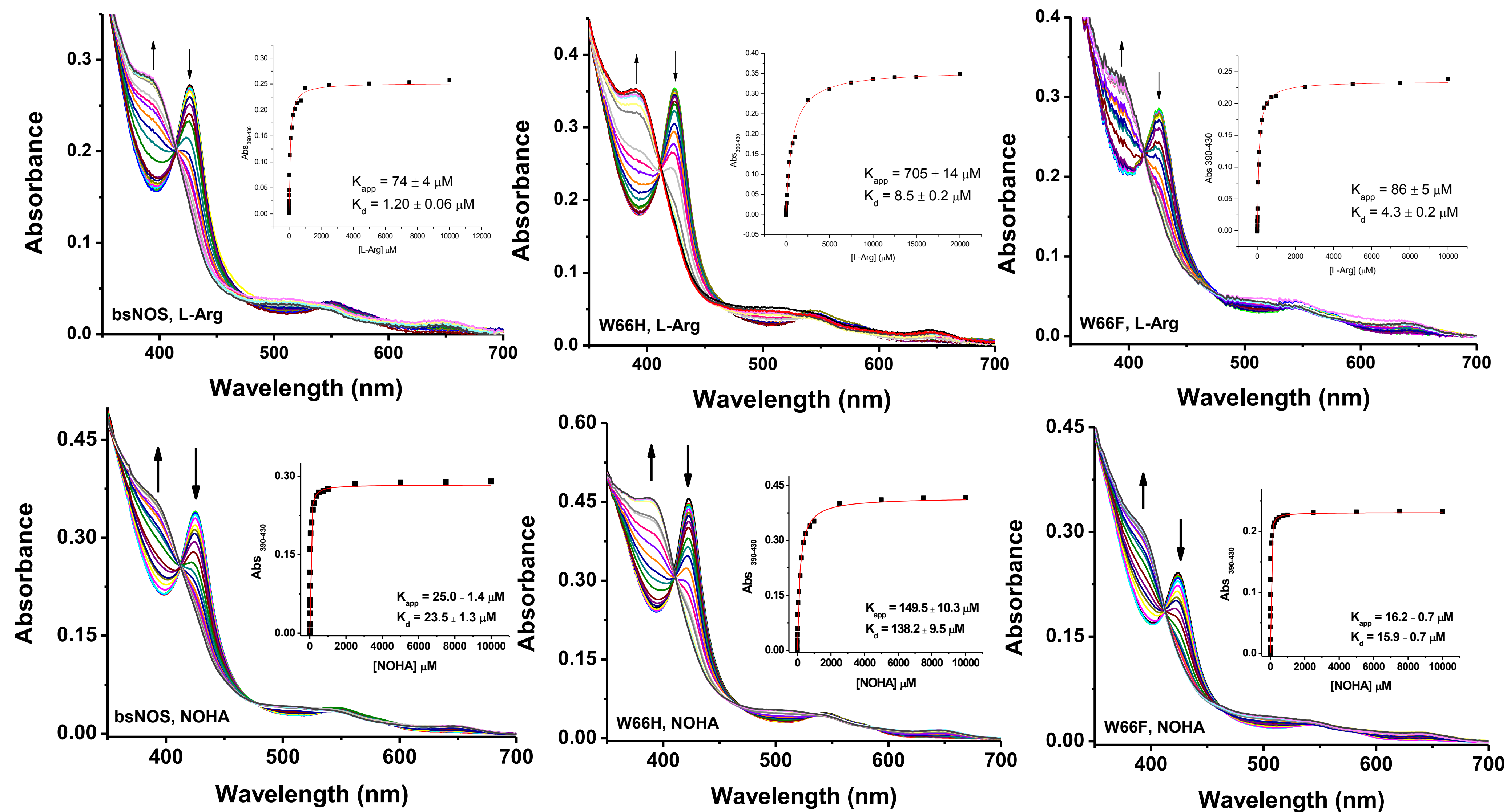

Figure S2. Spectrophotometric titrations of L-Arg (upper panels) and NOHA (lower panels) and determination of the binding affinity constants, K_d. W66H and W66F bsNOS display distinct substrate binding affinity with respect to wild type bsNOS. The titrations were performed in the presence of H_4T (50 μM), in buffer EPPS (40 mM, pH 7.60) containing 150 mM NaCl and 10% glycerol, at 25 ^oC. Binding constants for L-Arg and NOHA were determined in the presence of 10 mM imidazole

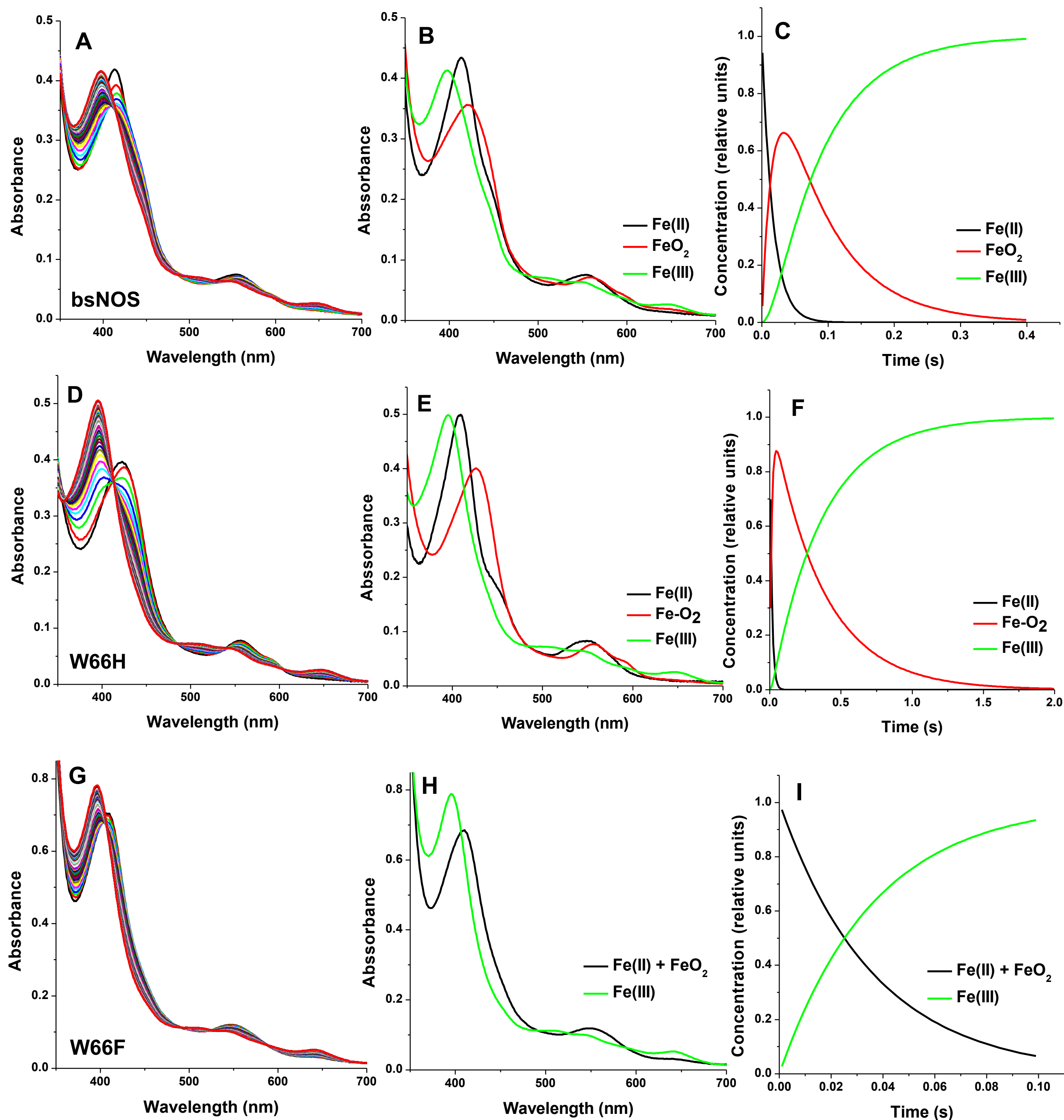

Figure S3. Single turnover reactions with L-Arg and H_4T of bsNOS wild type (A-C) and mutant proteins W66H (D-F) and W66F (G-I). Three species were identified by global analysis using the SpecFit 3.0 software: Fe(II) (Soret peak ~412 nm), Fe-O_2 (Soret peak ~ 427 nm) and Fe(III) (Soret peak ~ 394 nm). Mutation of Trp^{66} to His results in a slower conversion of the Fe(II)-O_2 species to form Fe(III) during single turnover reactions. Unlike the mammalian counterpart W188H iNOSoxy, decay of the Fe(II)-O_2 species in W66H occurs without the buildup of a detectable intermediate. Replacement of Trp^{66} by Phe results in a faster conversion of the Fe(II)-O_2 species to form Fe(III), as depicted by the time courses of the different species with respect to wild type bsNOS.

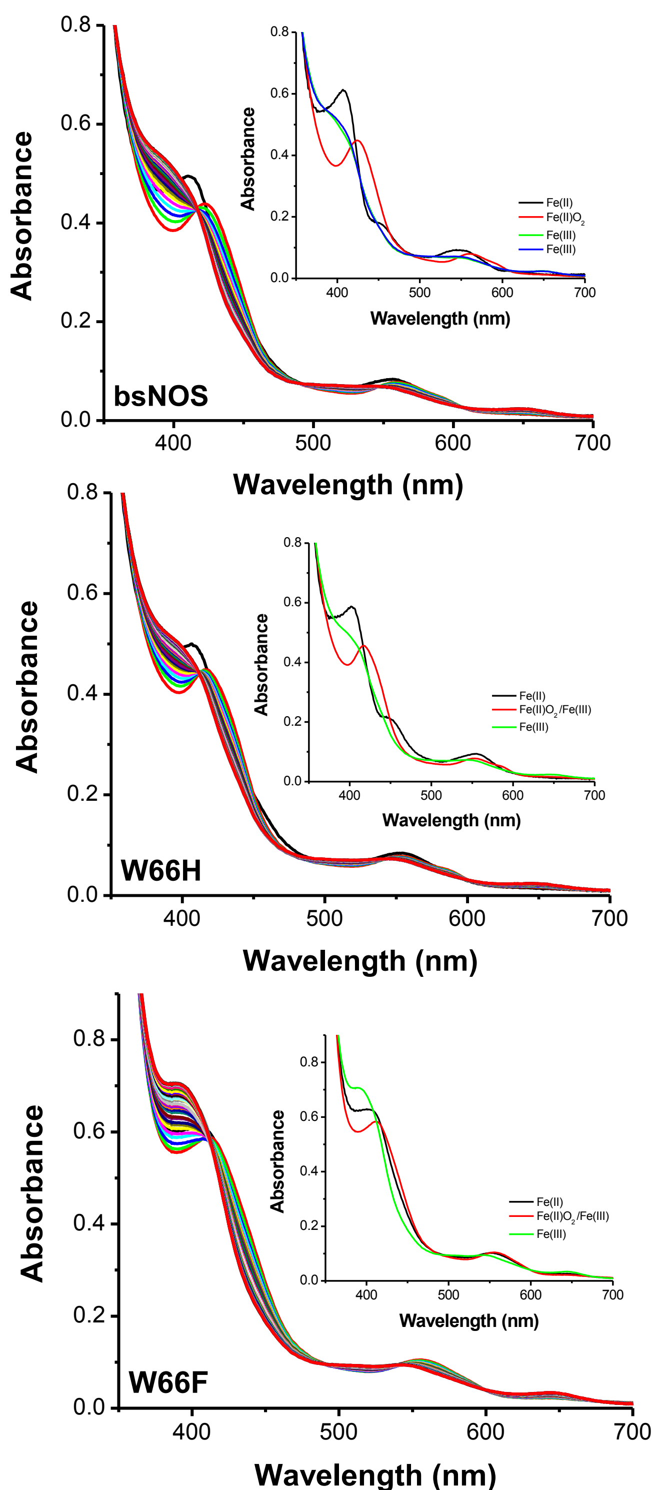

Figure S4. Single turnover reactions with NOHA and H_2T for bsNOS wild type, and mutant proteins W66H and W66F. Three species were identified by global analysis using the SpecFit 3.0 software: Fe(II) (Soret peak ~412 nm), Fe-O_2 (Soret peak ~ 427 nm) and Fe(III) (Soret peak ~ 394 nm). The reaction of wild type bsNOS shown herein was fit to a three-exponential model to evaluate/rule out the formation of an Fe(II)-NO complex. No evidence for Fe(II)-NO formation was found under these experimental conditions. Note: The marked absorption around 360-380 nm is due to the strong yellow color of H_2T.

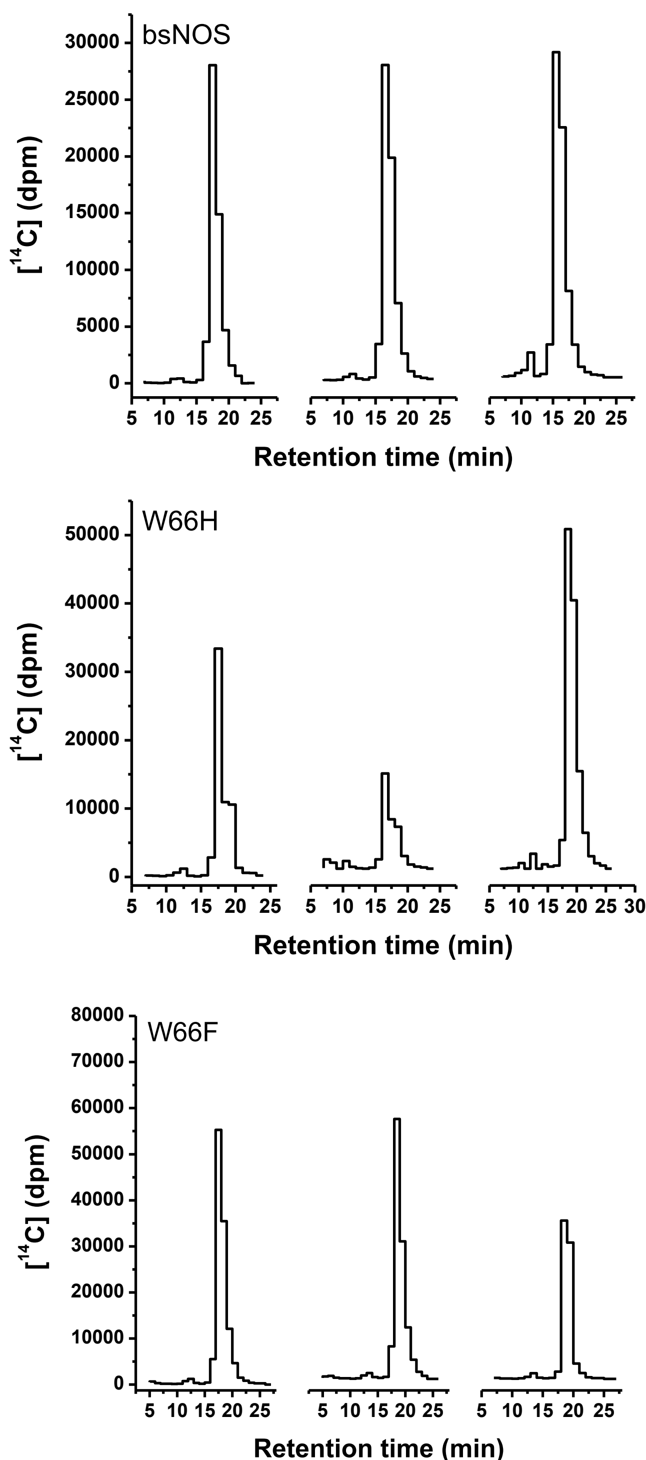

Figure S5. HPLC chromatograms for the formation of [^{14}C]-NOHA from [^{14}C]-L-Arg under single turnover conditions, at infinite time (10 min). Conversion of [^{14}C]-L-Arg to [^{14}C]-NOHA amounted to 2.71, 3.08 and 2.34 % of the total substrate, which resulted in a net conversion of 0.27, 0.31 and 0.23 NOHA per heme for bsNOS wt, W66H and W66F, respectively. The retention times for [^{14}C]-NOHA and [^{14}C]-L-Arg were ~13 and 18 min, respectively. Three independent samples were analyzed for each protein. The error associated with these measurements was less than 10%.

Table S1. UV-vis absorption maxima of bsNOS and mutants W66H and W66F reconstituted with H_4F/DTT (100 μM/300 μM) and L-Arg (1 mM) or imidazole (5 mM) in EPPS buffer (40 mM, pH 7.60) containing 150 mM NaCl and 10% glycerol.

	Soret (nm)	Visible (nm)
bsNOS, wt		
Fe(III)	394	509, 546, 649
Fe(III)-Imid	426	548
Fe(II)	412	554
Fe(II)-CO	446	554
Fe(II)-NO	438	567
W66H		
Fe(III)	392	506, 552, 648
Fe(III)-Imid	424	541
Fe(II)	406	555
Fe(II)-CO	440	554
Fe(II)-NO	435	562
W66F		
Fe(III)	394	506, 545, 643
Fe(III)-Imid	426	544
Fe(II)	409	552
Fe(II)-CO	449[a], 420	557
Fe(II)-NO	439	569

[a] The species at 449 nm shifts slowly to an uncharacterized species with absorption maximum at 420 nm.

Table S2. Observed rate constants for the reactions of bsNOS, W66H and W66F in the presence of H_2B with L-Arg as substrate.

	$Fe^{(II)} \xrightarrow{k1} FeO_2 \xrightarrow{k2} Fe^{(III)}$	
H_2B / L-Arg		
	k_1	k_2
bsNOS	>200	0.35
W66H	>200	0.34
W66F	>200	1.60

Table S3. Observed rate constants for the reactions of bsNOS, W66H and W66F in the presence of H_2T, with L-Arg or NOHA as substrates.

	$Fe^{(II)} \xrightarrow{k1} FeO_2 \xrightarrow{k2} Fe^{(III)}$	
H_2T / L-Arg [a]		
	k_1	k_2
bsNOS	55	0.42
W66H	39	0.12
W66F	102	2.8
H_2T / NOHA [b]		
	k_1	k_2
bsNOS	33	0.71
W66H	24	0.97
W66F	65	1.47

[a,b] Each value is an average of 5-10 measurements. The error associated to these measurements was 2-5 %.

Table S4. Rates of $Fe(II)O_2$ complex conversion to Fe(III) of selected hemeproteins.

Enzyme	**Temperature**	**k_{obs} (s^{-1})**	**Reference**
		$Fe(II)O_2$ decay	
iNOSoxy, H_2B	10 °C	0.3	(1)
iNOSoxy W188H, H_2B	10 °C	0.0044	(1)
nNOSoxy, no pterin	10 °C	0.14	(2)
drNOS L-Arg, no pterin	10 °C	1.37	(3)
drNOS, L-Arg, H_2T	10 °C	0.1	(4)
drNOS, NOHA, H_2T	10 °C	0.1	(4)
CYP101 w/ substrate	5-20 °C	0.0003-0.0043	(5)
CYP108 w/ substrae	4-20 °C	0.0007-0.017	(5)
CYP2A6 w/ substrate	23 °C	0.3	(6)
CYP2B4 w/ substrate	15 °C	0.09	(7)
CYP11A1 w/ substrate	4 °C	0.01	(8)
CYP3A4 w/ substrate	6 °C	0.37	(9)
P450 BM3, wild type	15 °C	0.14	(10)
P450 BM3, mutant F393H	15 °C	0.003	(10)
P450 BM3, mutant F393W	15 °C	0.54	(10)
P450 BM3, substrate-free	15 °C	0.089	(11)
P450 BM3, arachidonate	15 °C	0.059	(11)
Mutant F393H, substrate-free	15 °C	0.018	(11)
Mutant F393H, arachidonate	15 °C	0.0013	(11)

Table S5. Maximum yields for the conversions of L-Arg to NOHA and NOHA to citrulline at infinite time by wild type and mutant *B. subtilis* NOSs in reactions performed with H_4T.

	Yield (NOHA or citrulline per heme) [a]		
	bsNOS	W66H	W66F
L-Arg to NOHA	0.27	0.31	0.23
NOHA to citrulline	0.60	0.58	0.59

[a] Three independent reactions were carried out for each protein. The error associated to these measurements was 5-10%.

Table S6. Oxidation rates of Fe(III)-NO complexes of selected hemeproteins.

Enzyme	Temperature	k_{obs} (s^{-1})	Reference
		Fe(II)NO oxidation	
gsNOS NOHA/H_4B	4 °C	0.040	(12)
bsNOS W66H	10 °C	0.063	This work[a]
bsNOS	10 °C	0.092	This work[b]
dNOSoxy	10 °C	0.093	(13)
nNOSoxy	10 °C	0.19	(14)
eNOSoxy	10 °C	0.63	(15)
bsNOS W66F	10 °C	1.17	This work[c]
W409F nNOSoxy	10 °C	1.3	(14)
iNOSoxy	10 °C	3.11	(16)

[a, b, c] Each value is an average of 5-10 measurements. The error associated to these measurements was 2-5%.

Reference list

1. Tejero, J., Biswas, A., Wang, Z. Q., Page, R. C., Haque, M. M., Hemann, C., Zweier, J. L., Misra, S., and Stuehr, D. J. (2008) *J Biol Chem* **283**, 33498-33507
2. Abu-Soud, H. M., Gachhui, R., Raushel, F. M., and Stuehr, D. J. (1997) *J Biol Chem* **272**, 17349-17353
3. Adak, S., Bilwes, A. M., Panda, K., Hosfield, D., Aulak, K. S., McDonald, J. F., Tainer, J. A., Getzoff, E. D., Crane, B. R., and Stuehr, D. J. (2002) *Proc Natl Acad Sci U S A* **99**, 107-112
4. Reece, S. Y., Woodward, J. J., and Marletta, M. A. (2009) *Biochemistry* **48**, 5483-5491
5. Montgomery, H. J., Dupont, A. L., Leivo, H. E., and Guillemette, J. G. (2010) *Biochem Res Int* **2010**, 489892
6. Tejero, J., Biswas, A., Haque, M. M., Wang, Z. Q., Hemann, C., Varnado, C. L., Novince, Z., Hille, R., Goodwin, D. C., and Stuehr, D. J. (2010) *Biochem J* **433**, 163-174
7. Matsumura, H., Wakatabi, M., Omi, S., Ohtaki, A., Nakamura, N., Yohda, M., and Ohno, H. (2008) *Biochemistry* **47**, 4834-4842
8. Hume, R., Kelly, R. W., Taylor, P. L., and Boyd, G. S. (1984) *Eur J Biochem* **140**, 583-591
9. Denisov, I. G., Grinkova, Y. V., Baas, B. J., and Sligar, S. G. (2006) *J Biol Chem* **281**, 23313-23318
10. Ost, T. W., Clark, J., Mowat, C. G., Miles, C. S., Walkinshaw, M. D., Reid, G. A., Chapman, S. K., and Daff, S. (2003) *J Am Chem Soc* **125**, 15010-15020
11. Clark, J. P., Miles, C. S., Mowat, C. G., Walkinshaw, M. D., Reid, G. A., Daff, S. N., and Chapman, S. K. (2006) *J Inorg Biochem* **100**, 1075-1090
12. Sudhamsu, J., and Crane, B. R. (2006) *J Biol Chem* **281**, 9623-9632
13. Ray, S. S., Tejero, J., Wang, Z. Q., Dutta, T., Bhattacharjee, A., Regulski, M., Tully, T., Ghosh, S., and Stuehr, D. J. (2007) *Biochemistry* **46**, 11857-11864
14. Adak, S., Wang, Q., and Stuehr, D. J. (2000) *J Biol Chem* **275**, 17434-17439
15. Santolini, J., Meade, A. L., and Stuehr, D. J. (2001) *J Biol Chem* **276**, 48887-48898
16. Wang, Z. Q., Wei, C. C., and Stuehr, D. J. (2010) *J Inorg Biochem* **104**, 349-356