Regioselective nitration of tryptophan by a complex between bacterial nitric-oxide synthase and tryptophanyl-tRNA synthetase

Madhavan R. Buddha\textsuperscript{1}, Tao Tao\textsuperscript{2}, Ronald J. Parry\textsuperscript{2} and Brian R. Crane\textsuperscript{1}\textsuperscript{*}

\textsuperscript{1}Department of Chemistry and Chemical Biology, Cornell University Ithaca NY 14853
\textsuperscript{2}Department of Chemistry, Rice University, Houston, TX 77005.
\textsuperscript{*}To whom Correspondence should be addressed. Phone:607-257-2961, Fax: 607-255-1248,
Email:bc69@cornell.edu
RUNNING TITLE: Specific tryptophan nitration by bacterial NOS
SUMMARY
Bacterial nitric-oxide synthase proteins (NOSs) from certain Streptomyces strains have been shown to catalyze biosynthetic nitration of tryptophanyl moieties in vivo [Kers, J.A., Wach, M.J., Krasnoff, S.B., Cameron, K.D., Widom, J., Bukhaid, R.A., Gibson, D.M., and Crane, B.R., and Loria, R. (2004) Nature 429, 79-82]. We report that the complex between D. radiodurans NOS (deiNOS) and an unusual tryptophanyl tRNA synthetase (TrpRS II) catalyzes the regioselective nitration of tryptophan (Trp) at the 4-position. Unlike, non-enzymatic Trp nitration, and similar reactions catalyzed by globins and peroxidases, deiNOS only produces the otherwise unfavored 4-nitro Trp isomer. Although deiNOS alone will catalyze 4-nitro-Trp production, yields are significantly enhanced by TrpRS II and ATP. 4-nitro-Trp formation exhibits saturation behavior with Trp (but not tyrosine) and is completely inhibited by the addition of the mammalian NOS cofactor tetrahydrobiopterin (H₄B). Trp stimulates deiNOS oxidation of substrate L-arginine (Arg) to the same degree as H₄B. These observations are consistent with a mechanism where Trp, and possibly adenyl-Trp formed by TrpRS II, binds in the NOS pterin site, participates in Arg oxidation, and subsequently becomes nitrated at the 4-position.

INTRODUCTION
The generation and fate of reactive nitrogen species (RNS) are important to a variety of physiological processes that include pathogen cell death, general progression towards disease states, and stimulation of regulatory pathways (1-3). For example, nitrated aromatic protein residues mark the involvement of RNS in neurodegenerative diseases, atherosclerosis, infections, inflammation and cancer (1-3). Globins and peroxidases have been shown to catalyze complex reactions that can result in nitration of aromatic amino acids (3-7). In mammals, a primary source of RNS is nitric oxide (NO), which is largely produced by the three isoforms of nitric-oxide synthase (NOS) (8). The benefits and repercussions of generating NO in a cellular environment are complex and far from well understood (1-7).

Whereas mammalian NOS-mediated nitration events are generally non-specific, a bacterial nitric-oxide synthase protein has recently been shown to participate in a specific biosynthetic nitration reaction (9). Some pathogenic Streptomyces produce a family of unusual
phytotoxin dipeptides (derivatives of cyclo-[L-tryptophanyl-L-phenylalanyl]) called thaxtomin that contain a 4-nitro-tryptophanyl moiety (10). The transferable pathogenicity island that contains the genes responsible for thaxtomin biosynthesis also codes for a NOS (9). Genetic and isotope labeling studies have shown that the Streptomyces NOS nitrates thaxtomin (9). The chemical mechanism of a NOS-mediated nitration is intriguing because NO is unlikely to react directly with indole (11,12). Nevertheless, readily oxidized forms of NO, such as nitrosonium (NO⁺), peroxynitrite (ONOO⁻), nitrosonium (NO₂⁺), or nitrogen dioxide (NO₂) actively nitrate aromatic amino acids (11,12).

Another link between tryptophan metabolism and bacterial NOS comes from our recent finding that in D. radiodurans, an unusual tryptophanyl tRNA synthetase (TrpRS II) interacts with the D. radiodurans NOS (deiNOS)². In addition to catalyzing charging of tRNA^{Trp}, TrpRS II increases NOS solubility, affinity for substrate, and NO-synthase activity². Uncharacteristically, D. radiodurans has two TrpRS genes: the first has ~40% identity to other typical TrpRS sequences, the second, which interacts with NOS, has only ~28% identity to other TrpRS sequences. Type I tRNA-synthetases catalyze two reactions in the production of aminoacyl-tRNA (13). First, the amino acid is activated to the aminoacyl-5'-adenylate, which is then reacted with the 3'-acceptor stem of tRNA to form the aminoacyl-tRNA. Adenylation of aminoacids also precedes their condensation into natural products by non-ribosomal peptide synthases, such as the enzymes that produce thaxtomin (10). Thus, deiNOS and stNOS both associate, either physically or genetically, with enzymes capable of activating Trp for subsequent chemical coupling.

Bacterial NOSs are homologous to the mammalian oxygenase domain (mNOS_{OX}) but lack an associated reductase domain (NOS_{red}) (14-17). Compared to mNOSs, bacterial NOSs have many similar physical, chemical, and structural properties that include that ability to catalyze the formation of nitric oxide from Arg in a manner dependent on reduced pterins (either with tetrahydrobiopterin (H₄B) or with the related cofactor tetrahydrofolate (THF) (14-17). In mNOS, H₄B functions to rapidly supply an electron to the heme center during oxidation activation for conversion of Arg to the intermediate N⁶-hydroxy-L-arginine (NHA) and for
subsequent oxidation of NHA to citrulline and NO (8,18). Interestingly, *D. radiodurans* does not contain the enzymes necessary to synthesize H_4B, although the more common reduced pterin, THF, can activate deiNOS *in vitro* (15).

Herein, we report that the deiNOS-TrpRS II complex catalyzes the regioselective nitration of Trp at the 4-position. Unlike non-enzymatic nitration of Trp, as well as those reactions catalyzed by peroxidases and globins, no other nitrated Trp byproducts are formed in appreciable amounts. Furthermore, the nitration reaction has saturation kinetics and is completely inhibited by H_4B. Finally, Trp is shown to stimulate NO synthesis by deiNOS from Arg. These observations indicate that the nitration reaction occurs by Trp binding to a site that binds pterins in the mammalian enzymes.

**MATERIALS AND METHODS**

*Preparation of deiNOS and TrpRS II* -
DeiNOS, was cloned with a N-terminal His_6-affinity tag, expressed in *E. coli* BL21(DE3) cells and purified by nickel-NTA affinity chromatography (Qiagen) as previously described² (14). Notably, to improve solubility, deiNOS was eluted from the nickel column with 25 mM HEPES (7.5), 500 mM NaCl, 200 mM imidazole, 0.25 M sucrose and then rapidly washed 10 times in a centrprep concentrator with a solubilising buffer (SB) made of 50 mM TRIS (pH 7.5), 200 mM NaCl, 0.3 M L-arginine (Arg), 20 mM L-tryptophan. Under these conditions the protein could be concentrated to 40 mg/mL. TrpRS II was expressed, purified and concentrated up to 40 mg/mL². To obtain a 1:1 complex with deiNOS, 100 uL of deiNOS (1mM in SB) was mixed with 100 uL of TrpRS II (1mM in 50 mM TRIS (pH 7.5), 150 mM NaCl).

*Preparation of iNOS-reductase* - The iNOS-reductase construct was prepared from a plasmid containing cDNA for full-length iNOS using the polymerase chain reaction (PCR). Primers were made to amplify the final 621 residues of human iNOS-2A type 1, starting immediately following
the calmodulin binding domain (residue 533). The PCR product was cloned into pET28 (Novagen) and expressed in *E. coli* BL21 (DE3) cells. NOS$_{\text{red}}$ was purified using Ni-NTA metal-affinity chromatography under standard procedures.

*Synthesis of 4-nitro-Trp* - 4-Nitro-DL-tryptophan was synthesized from commercial 4-nitroindole by conversion of the latter to 4-nitrogramine using the procedure of Melhado and Brodsky (19). The 4-nitrogramine was then converted to the hydrochloride salt of 4-nitro-DL-tryptophan in three steps by modification of the procedure of Settimo (20). The free amino acid was obtained from the hydrochloride salt by cation-exchange chromatography.

*DeiNOS Nitration from hydrogen peroxide* - 50 µM deiNOS in SB was treated with 20 mM hydrogen peroxide and the reaction mix was monitored for 2 hrs at 22°C. Similarly, 50 µM complex prepared as mentioned above was treated with 20 mM hydrogen peroxide and incubated at 22°C for 2 hrs. Products of the reaction were analyzed by reverse-phase HPLC (see below) and progress of the reaction was followed by the increase in absorbance at 400 nm. Due to the relatively small extinction coefficient of nitro-Trp, approximately 10-50 times more deiNOS was used for nitration reactions that is typical for standard NOS assays. Nitro-Trp formation as a function of Trp concentration was carried out with peroxide, deiNOS and the TrpRS II-deiNOS complex exchanged in SB that did not contain tryptophan; final reaction mixtures were made up in buffers containing 50 mM TRIS (pH 7.5), 200 mM NaCl, 0.3 M Arg and increasing concentrations of tryptophan (1-20 mM). In the absence of sucrose/imidazole, deiNOS requires a high concentration of Arg for solubility. Progress of the nitration reaction was monitored by following the increase in absorbance at 400 nm and applying an extinction coefficient for nitro-Trp of $\varepsilon_{400} = 3000 \text{ M}^{-1}\text{cm}^{-1}$; $\varepsilon_{400} = 4200 \text{ M}^{-1}\text{cm}^{-1}$ was used to follow nitro-Tyr production (4). Because of the relatively large concentrations of deiNOS employed in the reactions, 0.2 cm cells were used to maintain an O.D. under 1.0 during kinetic assays.

*Nitration reaction from mammalian NOS reductase* -

For nitration reactions using mammalian reductase (mNOS$_{\text{red}}$) instead of peroxide, 50 µM
deiNOS or deiNOS-TrpRS II complex was treated with 10 µM iNOS reductase, 2 µM FAD, 10 µM NADPH, in presence or absence of 5-50 µM tetrahydrobiopterin (H4B) (K_M of H4B for deiNOS = 10 µM (14)) and in the presence and absence of 30 µM ATP.

Non-enzymatic nitration of tryptophan -
Hydrogen peroxide and nitrite were used to generate nitrating agents, which primarily include peroxynitrite (4,21). Hydrogen peroxide (10 mM in 0.1M potassium phosphate buffer, pH 6.9) was treated with sodium nitrite (10 mM in 0.1M potassium phosphate buffer, pH 6.9) and vortexed for about 1 min. The vortexed mixture was added to L-tryptophan (10 mM) and the reaction mixture was incubated at 37°C for about 1 hr and the products were evaluated using reverse-phase HPLC, mass-spectroscopy and UV-Visible spectroscopy.

Purification of nitration products - The reaction mixture was passed through Supelco’s Bio Wide Pore C18-5 column (25 cm X 4mm) with pore size of 300 Å. A gradient was run using buffers A (0.07 % TFA in water) and B (0.07 % TFA in acetonitrile) and the products collected, lyophilized and analyzed using Micromass ZMD 4000 mass spectrometer operated in the positive ion mode with a capillary voltage of 3000 V and cone voltage of 3.0 V.

NOS assays – Nitrite formation by deiNOS was monitored using the Griess reagents, whereas NO production was followed with the oxy-haemoglobin assay (14,22).

RESULTS

Production of 4-Nitro-tryptophan by deiNOS-TrpRS II -

_D. radiodurans_ NOS catalyzes the production of nitro-tryptophan and nitrite/nitrate from Arg or NHA in the absence or presence of TrpRS II (Table 1). The reaction requires a source of reduced oxygen, which can either be supplied as peroxide or as molecular oxygen and a reductase protein. As has been demonstrated for mNOS under some conditions (22), deiNOS will catalyze nitrite production from Arg and peroxide (Table 1). Nitrite is an end product of NO or related
species (e.g. nitroxyl, nitrosium, collectively hereafter referred to as [NO]) reacting in oxygenated solution and a typical marker of NOS activity. As a reductase partner for deiNOS has yet to be identified, we employed a surrogate mammalian NOS_{red} and NADPH to drive the reaction in the absence of peroxide (14) (Table 1). Human inducible NOS_{red} was chosen as the best electron donor after also testing rat neuronal NOS_{red} and four other homologous multi-flavin reductases from \textit{B. subtilis} and \textit{E. coli}. Products were analyzed by HPLC (Fig. 1A), mass-spectroscopy (Fig. 1B), and optical spectroscopy (Fig. 1B). Under all conditions tested, the reaction produced one nitro-Trp isomer, which elutes on a reverse-phase HPLC column at the same retention time as a synthetic 4-nitro-Trp standard (Fig. 1A). Isomers of nitro-Trp can be readily differentiated by their chromatographic behavior and their far-UV/visible absorption spectra (4,5). The product was further confirmed to be 4-nitro-Trp by its characteristic absorption spectrum (Fig. 1B) and mass spectrum (Fig. 1B), whose parent ion and fragments also match the 4-nitro-Trp standard. Consistent with previous reports of non-specific Trp nitration (4-6,21) reactions of Trp with nitrite and peroxide (a peroxynitrite generating system (4,21)) produced primarily a product whose much longer HPLC retention time and mass-spectrum identify it as N^{1}-nitro-Trp (not shown), and a minor product (Fig. 1A) with a mass and elution profile consistent with the favored 6-nitro Trp isomer (4,5). Comparisons between HPLC elution areas of the 4-nitro-Trp product and absorption changes in the reaction mixture indicated that 4-nitro-Trp formation accounted for at least 80% of the absorbance change at 400 nm (\Delta A_{400}). No other small molecule products with far UV/visible absorption were observed in appreciable quantities. Thus, kinetics of Trp nitration were followed by monitoring (\Delta A_{400}). Addition of 50 U/mL of superoxide dismutase also did not significantly reduce the production of nitro-Trp by deiNOS in either the reaction with peroxide or with mNOS_{red}.

\textit{Stimulation of 4-nitro formation by TrpRS II and ATP -}

Addition of TrpRS II stimulates the production of 4-nitro Trp by a factor of 2-3 whether peroxide
or NOS$_{\text{red}}$/NADPH drives the reaction (Table 1). In parallel studies, we find that TrpRS II increases the affinity of deiNOS for Arg and stimulates nitrite production from NHA in oxygenated solution$^2$. Surprisingly, the peroxide catalyzed Trp nitration reaction enhances further by the addition of ATP (Table 1). Consistent with greater incorporation of [NO] into the 4-nitro-Trp product, the amount of nitrite produced decreases as the amount of 4-nitro-Trp increases when ATP is present. ATP has no effect on 4-nitro-Trp or [NO] formation by deiNOS alone. ATP binds tightly to TrpRS II$^2$ and is required for Trp-adenylation by the synthetase.

The effects of pterin on 4-nitro-Trp and NO production -
Trp acts analogously to H$_4$B in the deiNOS catalyzed nitration reaction. Similar to mNOS (22), production of [NO] from Arg and mNOS$_{\text{red}}$ by deiNOS enhances ~10X by H$_4$B (Table 1). This is consistent with the role of H$_4$B in donating an electron to the NOS heme center during oxygen activation (8,18). Surprisingly, in the absence of H$_4$B, but presence of Trp, nitrite is still produced from Arg at nearly equal amounts (despite 4-nitro-Trp also being formed) (Table 1). Notably, in the presence of Trp, H$_4$B allows nitrite production, but inhibits 4-nitro-Trp formation with increasing concentration until no 4-nitro-Trp is formed at an H$_4$B:deiNOS-TrpRS II stoichiometry of 1:1 (Table 1). H$_4$B does not reduce the amount of nitrated product (A$_{400}$) in the non-enzymatic nitrite/peroxide reaction, nor does it affect nitrite formation by deiNOS in the presence of Trp. Thus, the ability of H$_4$B to inhibit nitro-Trp formation but not nitrite formation suggests that Trp binds in the deiNOS pterin site and furthermore can stimulate oxidation of Arg from this position. We were unable to detect NO production from deiNOS-TrpRS II in the presence of Trp by the oxy-haemoglobin assay; however, the amount of NO expected from the low deiNOS concentrations required to monitor oxy-haemoglobin conversion were at the detection limit. NO production from Trp is of interest because H$_4$B-depleted mNOS will still produce nitrite in oxygenated solution but does so through nitroxyl (NO$^-$), rather than NO formation (22,23).
Trp nitration by deiNOS exhibits saturation behavior -

The dependence of 4-nitro Trp formation by deiNOS on Trp concentration exhibits saturation behavior with an observed Michaelis constant ($K_M$) of 2.8 +/- 0.6 mM (Fig. 2). Addition of TrpRS II increases the effective $V_{\text{max}}$ 2-3 times and decreases $K_M$ to 1.5 +/- 0.5 mM (Fig. 2). Such saturation kinetics also indicate that 4-nitro-Trp production involves Trp binding to deiNOS. A different concentration dependence would be expected for reaction of free Trp with a diffusing RNS generated by deiNOS. We also tested inhibition of Trp nitration by the peroxynitrite scavenger cysteine (21). Although 1 mM cysteine completely prevented nitro-Trp formation from nitrite and peroxide, 4-nitro-Trp formation by deiNOS decreased only slightly (15%) in the presence of cysteine (Table 1). Tyrosine, the more reactive amino acid for nitration (24), is also nitrated by deiNOS, but with lower turnover numbers than Trp. At the Tyr concentrations we could evaluate, the reaction did not saturate and appeared to be roughly first order (Fig. 2).

DISCUSSION

The regioselective nitration reaction catalyzed by deiNOS that produces only 4-nitro-Trp is in marked contrast to non-enzymatic and heme protein catalyzed Trp nitration reactions. Agents capable of nitrating aromatic aminoacids include peroxynitrite, nitrous acid, nitrite and hydrogen peroxide, nitric oxide and dioxygen, and acyl nitrates (4,11,12,24). Moreover, peroxidases in the presence of nitrite and H$_2$O$_2$, and myoglobin in the presence of nitrite, NO or O$_2$ are well characterized in their ability to nitrate Trp (4-6,21,25,26). These reactions generate a large number of products which include the 4-nitro, 5-nitro, 6-nitro, 7-nitro, N$^1$-nitro- and N$^1$-nitroso- derivatives of Trp as well as hydroxylated Trp and N-formylkinureine (4-6,21,25,26). Trp nitration reactions catalyzed by hemeproteins involve multiple competing reaction pathways; of these, peroxynitrite intermediates are often involved, as are indole radicals generated from Trp oxidation by compound I or II peroxidase species (4,11,12,24). Given the non-specific
character of these reactions, it is then remarkable that deiNOS produces a single nitrated
derivative of Trp, the 4-nitro isomer. Interestingly, this is the same nitro-Trp derivative produced
by the Streptomyces NOS in the thaxtomin phytotoxin. Due to the preferential reactivity of other
positions on the indole ring, deiNOS likely interacts with Trp in order to protect more reactive
sites from modification. Interestingly, the mammalian NOS cofactor H$_4$B does not significantly
affect [NO] production by deiNOS in the peroxide assay but completely inhibits production of
nitro-Trp. This result indicates that Trp nitration by deiNOS does not occur by reaction between
free Trp and a diffusible nitrating agent derived from deiNOS and Arg. Furthermore, the inability
of the peroxynitrite scavenger cysteine to prevent nitro-Trp formation rules out the obligatory
participation of free preoxynitrite.

Saturation behavior of the nitration reaction with Trp concentration also supports Trp
binding to deiNOS. Neither saturation kinetics nor comparably high product yields are
observed with Tyr, which is generally at least as reactive towards nitrating agents as Trp (24).
Inhibition of 4-nitro Trp formation by H$_4$B indicates that rates saturate with Trp concentration
because Trp binds in the pterin site. In bsNOS, H$_4$B accelerates decay of the heme ferrous-oxy
species by presumably acting as a rapid electron donor to heme, akin to its role in mammalian
NOS catalysis (8,17,18,27). The dependence of deiNOS on reduced pterins (15) or Trp for [NO]
formation from Arg could imply that deiNOS maintains this mechanism, but that Trp can
substitute for H$_4$B as an electron donor. This is surprising, given that the reduction potential of
the Trp radical (1.0 V, (28)) is expected to be much higher than that for H$_3$B (0.15 - 0.3 V, (29)
and references therein). Nonetheless, the protein and or protonation environment could
modulate these potentials (27) and indeed, NOSs do provide a negative electrostatic field at the
pterin site that will facilitate oxidation of cofactors (18). Alternatively, Trp could be oxidized by
the compound I– like oxidation state of NOS, which follows reaction of two-electron reduced
oxygen (or peroxide) at the heme center. Peroxidases are capable of this reaction (4), but it is
difficult to rationalize why Trp stimulates nitrite production from deiNOS-NOS$_{red}$ if Trp
participation in the reaction is limited to steps after oxygen activation.
Coupling oxidation of Trp to the heme chemistry that oxidizes Arg to NO is an attractive means of providing a reactive indole radical to the [NO] species derived at the heme site. Such a mechanism would limit the toxic effects of [NO] diffusion away from deiNOS. If [NO] coupling with the radical proceeded prior to [NO] reaction with oxygen, a 4-nitroso-Trp would be a likely initial product (Fig. 3). As C-nitroso-Trp may be unstable to both oxidation and nitroso-migration (24,30), it is not surprising no such species was observed.

TrpRS II activates deiNOS for production of [NO] and for production of 4-nitro-Trp. We have found that these proteins tightly associate in vitro and in cell lysates of D. radiodurans2. Significantly, TrpRS II activation of deiNOS nitration increases in the presence of ATP. TrpRS II requires ATP to adenylate Trp prior to tRNA charging. deiNOS itself does not interact with ATP, nor does ATP affect nitro-Trp or [NO] production of deiNOS alone. Thus, 5'-adenyl-Trp, produced by TrpRS II may be a preferred substrate for deiNOS nitration (Fig. 3). The 5' adenyl-Trp is unstable to hydrolysis and difficult to isolate as an intermediate (13). More productive binding of 5'-adenylate compared to Trp may explain the apparent activation seen with ATP. Alternatively, ATP binding to TrpRS II may influence deiNOS in a manner that stimulates Trp nitration. The ability of TrpRS II and ATP to enhance the deiNOS nitration reaction underscores the functional coupling of these two enzymes.

Many enzymes involved in secondary metabolism, such as non ribosomal peptide synthases, polyketide synthases, terpene synthases and taxadiene synthases have relatively slow turnover numbers that often range between 0.001-0.8 min⁻¹ (The Comprehensive Enzyme Information System, www.brenda.uni-koeln.de). Furthermore, enzymes that metabolize Trp such as tryptophan transaminase, peptide-tryptophan dioxygenase, tryptophanase and L-tryptophan oxidase, have $K_m$ values for Trp of ~1mM. Thus, the catalytic parameters for Trp nitration by deiNOS are well within the range expected for a biosynthetic function. The production of 4-nitro-Trp isomer by both deiNOS and the Streptomyces NOS that biosynthesizes thaxtomin may not be coincidental, particularly if both reactions require Trp binding to NOS proteins that do indeed share high homology in their pterin binding sites.
Acknowledgements – We thank Stuart Krasnoff for assistance with mass-spectrometry and Harold Scheraga and colleagues for help with HPLC. This work was supported by a Petroleum Research Funds grant 38374-AC (to B.R.C.) and a Robert A. Welch Foundation grant C-0729 (to R.J.P.)

FOOTNOTES:
1. The abbreviations used are:Arg, L-arginine; Cit, L-citrulline, NHA, Nοω-hydroxyl-L-arginine, NOS, nitric-oxide synthase; mNOS, mammalian NOS; deiNOS, Deinococcus radiodurans NOS; NOS\textsubscript{red}, NOS reductase domain; NOS\textsubscript{OX}, NOS oxygenase domain; H\textsubscript{4}B, (6R)-5,6,7,8-tetrahydro-L-biopterin; NO, nitric oxide; \([\text{NO}]\), nitric oxide, nitroxyl (NO\textsuperscript{-}) or nitrosium (NO\textsuperscript{+}); RNS, reactive nitrogen species; SB, solubilizing buffer; TFA, trifluoroacetic acid; Trp, L-tryptophan; TrpRS II, the non-standard tryptophanyl tRNA-synthetase of \textit{D. radiodurans}.
2. Buddha, M.R., Keery, K., and Crane, B.R. (2004) \textit{Proc. Natl. Acad. Sci. USA}, In Press.

REFERENCES
1. Beckman, J. S. (1996) \textit{Chem. Res. Toxicol.} \textbf{9}, 836-844
2. Ischiropoulos, H., (ed). (2002) \textit{Free Rad. Biol. Med.} \textbf{33}, 727-874.
3. Eiserich, J. P., Baldus, S., Brennan, M. L., Ma, W. X., Zhang, C. X., Tousson, A., Castro, L., Lusis, A. J., Nauseef, W. M., White, C. R., and Freeman, B. A. (2002) \textit{Science} \textbf{296}, 2391-2394
4. Sala, A., Stefania, N., Roncone, R., Casella, L., Monzani, E. (2004) \textit{Eur. J. Biochem.} \textbf{271}, 2841-2851
5. Herold, S., Shivashankar, K., and Mehl, M. (2002) \textit{Biochemistry} \textbf{41}, 13460-13472
6. Herold, S. (2004) \textit{Free Rad. Biol. Med.} \textbf{36}, 565-579
7. Brennan, M. L., Wu, W. J., Fu, X. M., Shen, Z. Z., Song, W., Frost, H., Vadseth, C., Narine, L., Lenkiewicz, E., Borchers, M. T., Lusis, A. J., Lee, J. J., Lee, N. A., Abu-Soud, H. M., Ischiropoulos, H., and Hazen, S. L. (2002) \textit{J. Biol. Chem.} \textbf{277}, 17415-17427
8. Stuehr, D. J., Santolini, J., Wang, Z., Wei, C., Adak, S. (2004) \textit{J. Biol. Chem.} \textbf{279},
9. Kers, J. A., Wach, M. J., Krasnoff, S. B., Widom, J., Cameron, K. D., Bukhalid, R. A., Gibson, D. M., Crane, B. R., and Loria, R. (2004) Nature \textbf{429}, 79-82

10. Healy, F. G., Wach, M., Krasnoff, S. B., Gibson, D. M., and Loria, R. (2000) Mol. Micro. \textbf{38}, 794-804

11. Koppenol, W. H. (1998) \textit{Free Rad. Biol. Med.} \textbf{25}, 385-391

12. Hughes, M. N. (1999) \textit{Biochim. Biophys. Acta} \textbf{1411}, 263-272

13. Retailleau, P., Yin, Y. H., Hu, M., Roach, J., Bricogne, G., Vonrhein, C., Roversi, P., Blanc, E., Sweet, R. M., and Carter, C. W. (2001) \textit{Acta Crystallogr. D} \textbf{57}, 1595-1608

14. 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) \textit{Proc. Nat. Acad. Sci. USA} \textbf{99}, 107-112

15. Pant, K., Bilwes, A. M., Adak, S., Stuehr, D. J., and Crane, B. R. (2002) \textit{Biochemistry} \textbf{41}, 11071-11079

16. Bird, L. E., Ren, J. S., Zhang, J. C., Foxwell, N., Hawkins, A. R., Charles, I. G., and Stammers, D. K. (2002) \textit{Structure} \textbf{10}, 1687-1696

17. Adak, S., Aulak, K., Stuehr, D.J. (2002) \textit{J. Biol. Chem.} \textbf{277}, 16167

18. Wei, C. C., Crane, B. R., and Stuehr, D. J. (2003) \textit{Chem. Rev.} \textbf{103}, 2365-2383

19. Melhado, L. L. a. B., J.L. (1988) \textit{J. Org. Chem.} \textbf{53}, 3852-3855

20. Da Settimo, A. (1962) \textit{Ann. Chim. (Rome)} \textbf{52}, 17-24

21. Alvarez, B., Rubbo, H., Kirk, M., Barnes, S., Freeman, B.A., Radi, R. (1996) \textit{Chem. Res. Toxicol.} \textbf{9}, 390-396

22. Adak, W., Q., and Stuehr, D.J. (2000) \textit{J. Biol. Chem.} \textbf{275}, 33554-33561

23. Rusche, K. M., Spiering, M.M., Marletta, M.A. (1998) \textit{Biochemistry} \textbf{37}, 15503-15512

24. Bonnet, R. a. N., P. (1977) \textit{Heterocycles} \textbf{7}, 637-659

25. Kato, Y., Kawakishi, S., Aoki, T., Itakura, K., Osawa, T. (1997) \textit{Biochem. Biophys. Res. Commun.} \textbf{234}, 82-84

26. Padmaya, S., Ramazenian, M.S., Bonds, P.L., Koppenol, W.H. (1996) \textit{Redox Report} \textbf{2}, 173-177.

27. Sorlie, M., Gorren, A. C. F., Marchal, S., Shimizu, T., Lange, R., Andersson, K. K., and
28. Tommos, C., Skalicky, J.J., Pilloud, D.L., Wand, A.J., Dutton, P.L. (1999) *Biochemistry* 38, 9495-9507

29. Gorren, A. C. F., Kungl, A. J., Schmidt, K., Werner, E. R., and Mayer, B. (2001) *Nitric Oxide - Biol. Chem.* 5, 176-186

30. Castro, A., Iglesias, E., Leis, J.R., Pena, M.E., Tato, J.V., Williams, D.L.H. (1986) *J. Chem. Soc. Perkin Trans. II* 2, 1165-1168

**FIGURE LEGENDS**

Fig 1. *D. radiodurans* NOS only produces the 4-nitro isomer of nitro-Trp. A) Reverse phase HPLC elution profiles comparing C-nitro Trp derivatives obtained from the nitration reaction of deiNOS-TrpRS II (green), peroxynitrite (nitrite + H2O2) (red), and the standard (L)4-nitrotryptophan (black). In the non-enzymatic reaction the major product, N1-nitro-Trp, elutes 36.5 min. No other products absorbing at 400 nm are formed by deiNOS-TrpRS II. B) Identification of the major nitration product of deiNOS as 4-nitro-Trp. ESI-Mass spectrum of the same product of the deiNOS-TrpRS II complex. Molecular ion (MH+, m/z = 250.0), and all major fragments (m/z = 233, 224, 299 and 199, 171) match with the 4-nitro-Trp standard. Inset: UV-visible spectrum of the major deiNOS-TrpRS II product after HPLC purification shows a 410 nm broad absorption band that is diagnostic for the 4-nitro Trp isomer.

Fig 2. Initial Trp nitration rates with deiNOS saturate with Trp concentration. Amount of 4-nitro-Trp in t = 12 min as a function of tryptophan concentration in presence of deiNOS (open circles) or the deiNOS-TrpRS II complex (filled circles). Data fit to a Michaelis saturation function yield an apparent $K_M$ for deiNOS of 1.5 +/- 0.5 mM, and for deiNOS-TrpRS II of 2.8 +/- 0.6 mM. Nitration of tyrosine (open triangles) occurs at lower yield and does not appear to follow saturation kinetics.
Fig 3. Schematic summarizing (A) non-enzymatic and (B) deiNOS-TrpRS II catalyzed Trp nitration. The tentative participation of an adenyl-Trp species in the enzymatic reaction is consistent with ATP-activation of product yields; participation of a nitroso-Trp intermediate is speculative.
Table I. Formation of nitro-tryptophan and nitrite by deiNOS under various conditions

| Reaction Monitored<sup>a</sup> | $k_{cat}$ (4-Nitro tryptophan) X 100 min<sup>-1</sup> | $k_{cat}$ (Nitrite) X 100 min<sup>-1</sup> |
|-------------------------------|-------------------------------------------------|-------------------------------------|
| DeiNOS + mRed<sup>b</sup>     | 1.5 +/- 0.15                                    | 2.3 +/- 0.5                         |
| DeiNOS + TrpRS II + mRed - Trp| ND<sup>c</sup>                                   | 0.5 +/- 0.2                         |
| DeiNOS + TrpRS II + mRed + H4B<sup>d</sup> - Trp | ND                                             | 4.5 +/- 0.5                         |
| DeiNOS + TrpRS II + mRed      | 3.2 +/- 0.5                                     | 5.0 +/- 0.5                         |
| DeiNOS + mRed + H4B<sup>d</sup> | ND                                             | 2.5 +/- 0.7                         |
| DeiNOS + TrpRS II + 5 µM H4B + mRed | 2.5 +/- 0.2                                 | 4.8 +/- 0.9                         |
| DeiNOS + TrpRS II + 25 µM H4B + mRed | 0.3 +/- 0.01                                 | 4.3 +/- 0.5                         |
| DeiNOS + TrpRS II + 50 µM H4B + mRed | ND                                             | 4.9 +/- 0.6                         |
| DeiNOS + TrpRS II + ATP + mRed | 5.1 +/- 0.4                                     | 2.3 +/- 0.7                         |
| DeiNOS + H2O2                 | 3.5 +/- 0.8                                     | 7.5 +/- 0.5                         |
| DeiNOS + TrpRS II + H2O2      | 11.0 +/- 3.0                                    | 15.0 +/- 5.0                        |
| DeiNOS + ATP + H2O2           | 2.0 +/- 0.8                                     | 7.1 +/- 0.6                         |
| DeiNOS + TrpRS II + ATP + H2O2| 25.0 +/- 7.0                                    | 6.9 +/- 0.5                         |
| DeiNOS + H2O2 + L-cysteine    | 2.9 +/- 0.6                                     | 7.0 +/- 0.6                         |

<sup>a</sup>Unless indicated, all reactions were carried out in the presence of 20 mM Trp at 22°C.

<sup>b</sup>Mammalian human inducible NOS reductase domain and NADPH.

<sup>c</sup>Not Detectable.

<sup>d</sup>50 µM H4B
Regioselective nitration of tryptophan by a complex between bacterial nitric-oxide synthase and tryptophanyl-tRNA synthetase
Madhavan R. Buddha, Tao Tao, Ronald J. Parry and Brian R. Crane

J. Biol. Chem. published online October 5, 2004

Access the most updated version of this article at doi: 10.1074/jbc.C400418200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

Supplemental material:
http://www.jbc.org/content/suppl/2004/10/26/C400418200.DC1