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Role of nitric oxide synthase in insect cell radioresistance: an *in-silico* analysis

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received May 23, 2008; revised July 07, 2008; accepted July 20, 2008; published September 02, 2008

Abstract:
Previous studies on various insect cell lines have displayed very high radioresistance in Lepidoptera (butterflies and moths) as compared to mammals as well as other orders of Insecta including Diptera. Since NOS is known to modulate cellular radiation sensitivity, we carried out *in silico* analysis of Lepidopteran NOS and compared its structural and functional features including the sequence homology, predicted tertiary structure, post-translational phosphorylation and intracellular localization with the other species. Our study demonstrates that Lepidopteran NOS, while carrying significant sequence homology with mammalian nNOS, has structural/ functional features that may enhance resistance to radiation and other stress agents. A higher phosphorylation score of Lepidopteran NOS (0.885±0.02 as against 0.694±0.094 of mammalian NOS; predicted using Net Phos 2.0) was observed at many well-conserved phosphorylation sites, which may reduce NOS activation by stress agents including radiation. Further, the primarily cytoplasmic localization of Lepidopteran NOS (score 23 against 10 of mammalian NOS, derived using WoLFPSORT), aided by higher phosphorylation scores as well as sequence-driven cytoplasmic localizing signals, may significantly reduce amplification of extraneous oxidative damage. Based on these findings, we hypothesize that a primarily cytosolic and less responsive NOS could significantly contribute to radioresistance of Lepidopteran insects as well as their cultured cell lines.

Keywords: nitric oxide (NO); nitric oxide synthase (NOS); multiple sequence alignment (MSA); phosphorylation mapping; phosphorylation score; sub-cellular localization

Background:
Nitric oxide (NO) is a biological effector molecule that acts as a multifunctional messenger in mammalian physiology such as vasodilatation, neurotransmission, cell-cell interaction, regulation of vascular tone and macrophage-mediated cytotoxicity. In the invertebrates such as insects (e.g., Drosophila), NO has been shown to be involved in imaginal disc development, synaptogenesis, formation of retinal projection pattern and behavioral responses. In addition to its established physiological roles, NO is also known to modulate various cellular responses to stressors like radiation, bacterial infection and hypoxic environment etc. NO is synthesized inside the cells through enzymatic conversion of L-Arginine to Citrulline catalyzed by a family of enzymes called NO synthases (NOS, EC 1.14.13.39). NOS like enzymes are coded in the genomes of most of the organisms including mammals, invertebrates and plants, albeit in varying isoforms. Three NOS isoforms are present in mammals, i.e., n-NOS (Neuronal Nitric oxide synthase), i-NOS (Inducible Nitric oxide synthase) and e-NOS (Endothelial Nitric oxide Synthase). Neuronal and endothelial NOS enzymes are produced constitutively and their activity is Ca^{++}-calmodulin (CaM) dependent. Inducible NOS is constitutively expressed at low level, and is induced by various stimuli like γ-interferon, LPS, ionizing radiation. However, its functioning does not depend on calcium, unlike the other two isoforms **[1, 2, 3]**. The core region of NOS oxygenase domain contains sub-domains for binding of heme, tertahydrobiopterin (H_4B) and arginine, which constitute the active site where NO synthesis occurs. The C-terminal reductase domain starts at the end of the CaM binding sequence and contains sub-domains for binding of FMN, FAD and NADPH. During NO synthesis, the reductase domain acquires electrons from NADPH and transfer them to the heme iron, which in turn binds and activates O_2 and catalyses NO synthesis. In e-NOS and n-NOS isozymes, the flavin to heme electron transfer is triggered by CaM binding, which explains how Ca^{++} and CaM can regulate NO synthesis by these constitutive NOS isoforms. All these NOS isoforms are homodimers in their active form and the reductase domain of one NOS polypeptide can transfer electrons to the oxygenase domain of another NOS polypeptide within a homodimer **[4, 5]**. Catalytic activity of NOS is known to be regulated through feedback inhibition by NO, H_4B level, interaction between NOS and NOSIPs (Nitric Oxide Synthase interacting Proteins) as well as via covalent modifications. NOS's have been reported to be myristoylated, palmitoylated, farnesylated, acetylated and phosphorylated in the oxygenase domain. Out of all these modifications, phosphorylation is known to lower the enzymatic activity whereas other modifications evidently play a role in the

ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 3(1): 8-13 (2008)

localization of enzyme to cytosol, plasma membrane and other organelles **[6, 7]**.

While mammals express all the three NOS isoforms, most of the invertebrates contain only one isozyme that has high homology with the mammalian nNOS. Importantly, both the endogenous (eNOS, nNOS) and inducible (iNOS) isozyme activities are known to modulate cellular radiation sensitivity **[8]**. Therefore, the presence of only one ortholog may possibly influence radiation response of invertebrates, including certain orders of class Insecta that generally display very high radioresistance. Insects remain very important model organisms for radiation response studies due to being phylogenetically closer to mammals compared to the other radioresistant organisms such as bacteria, yeast and nematodes **[9, 10, 11]**. The general stress resistance of insect cells may be contributed by differences in the regulation of NOS as well as their cellular localization and post-translational modification, besides other known cellular mechanisms. Therefore, in this in-silico study we compared the structural and functional features of insect and mammalian NOS isoforms that can play an important role in stress response, including the post-translational modifications and cellular localization. Homology analysis as well as the predicted tertiary structures, phosphorylation sites and cellular localization status of Lepidopteran insect orthologues show peculiar nature of NOS in various insect species, which is likely to contribute to their radioresistance.

Methodology:

Dataset and sequence analysis

Global PSI-BLAST (www.ebi.ac.uk/blastpgp/index.html) search was performed using NOS protein sequence of *Bombyx mori* (Lepidoptera) and a list of all evolutionarily related proteins was created. From this list, full-length sequences for orthologues from 22 organisms representing various orders spread over all phyla were retrieved using GeneBank **[12]**, with protein name and species (Figure 1) or accession numbers as keywords. All these sequences were subjected to MSA for studying the sequence conservation within functional domains of nitric oxide synthase (NOS) as well as for residues that are required for substrate binding, dimerization and post-translational phosphorylation. MSA was carried out using CLUSTAL W **[13]**, and the aligned sequences were represented in background RASMOL colouring using the CLC Free Workbench version 4.5.1.

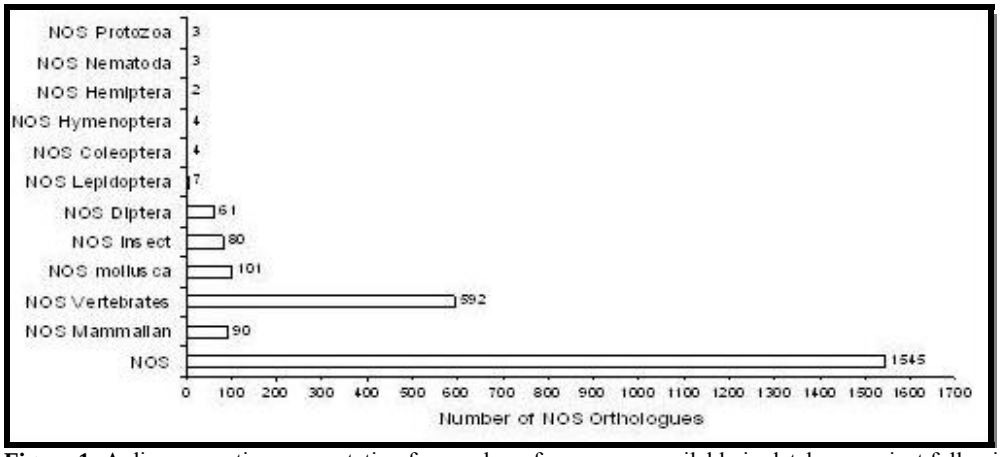

Figure 1: A diagrammatic representation for number of sequences available in database against following keywords used.

Prediction of insect NOS three-dimensional tertiary structure

The tertiary structure of insect (*Drosophila* and *Bombyx*) NOS oxygenase and reductase domains was predicted using PDB entries derived from crystal structure data of mammalian (Rat) nNOS oxygenase and reductase domain. All structure prediction was carried out using SWISS MODEL SERVER (http://swissmodel.expasy.org/workspace) in automated mode. Further, PDB files predicted for insect NOS were visualized using the DeepViewer Software. Different secondary structures like helixes, β-Sheets and coils are presented in orange, green and blue color, respectively (Figure 2). Residues important for zinc and arginine binding are also indicated in the three-dimensional folded structure of NOS oxygenase domain.

Prediction of post-translational phosphorylation and sub-cellular localization

Prediction for serine, threonine and tyrosine phosphorylation sites were done using Net Phos 2.0 server (http://www.cbs.dtu.dk/services/NetPhos). Putative protein sequences for each homologue were given as input, and default settings were used to predict the phosphorylation score. Prediction for relative abundance of various NOS homologues based on their protein sequence in different sub-cellular compartments was done using WoLFPSORT (http://wolfpsort.org/).

ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 3(1): 8-13 (2008)

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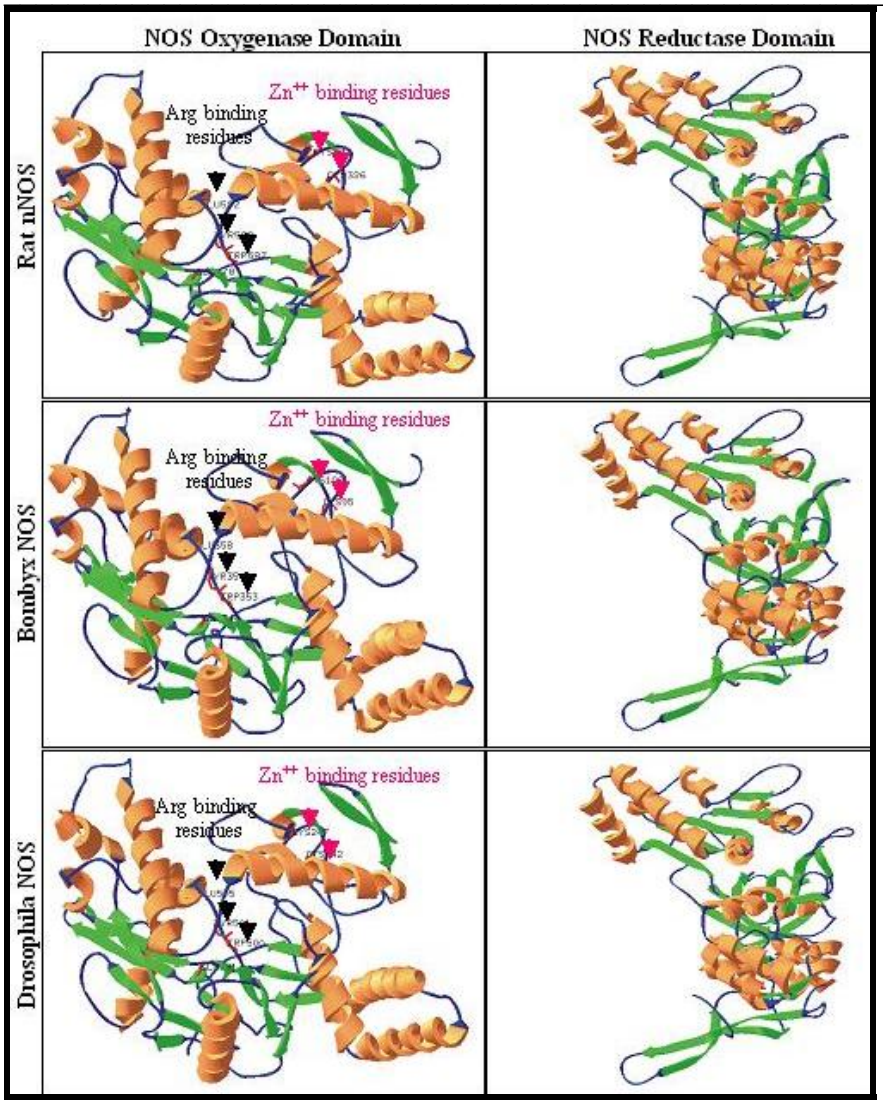

Figure 2: Predicted tertiary structure of Dipteran (*Drosophila*) and Lepidopteran (*Bombyx*) NOS on the basis of PDB files derived from rat nNOS crystal structure data. Separate oxygenase and reductase domains are shown with their reference rat nNOS structure. All structures are represented in ribbon model. Helixes, β-sheets and coils are shown in orange, green and blue colours, respectively.

Discussion:

Insect NOS is structural and functional homologue of mammalian nNOS

All NOS orthologues from higher invertebrates show a high degree of homology with the mammalian eNOS and nNOS, as discussed above. Whole genome sequencing of organisms like *C. elegans* and *D. melanogaster* has already indicated the presence of only one NOS protein, which has similarity with the constitutive nNOS of mammalian system **[14, 15, 16]**. Comparison of the amino acid sequence of insect (*Drosophila* and *Bombyx*) NOS domains (Figure 3) or whole protein (data not shown) with sequences of mammalian NOS isozymes using PSI-BLAST revealed the maximum identity with mammalian nNOS.

In addition to these domains, amino acids relevant for Zn++ binding (C415, C420 alignment position) and for arginine binding (Q568, W677 and Y678 alignment position) are highly conserved in all species as evident from the Figure 3. PKA phosphorylation site S464 is also conserved between constitutive mammalian NOS and insect NOS (Figure 3). Prediction of phosphorylation sites within the oxygenase domain of *Drosophila* and *Bombyx* NOS also indicates that the sites are well conserved (Table 1 under supplementary material). In addition to these conserved sites, the other sites responsible for electron transport, which are present in the reductase domain are also conserved among all NOS orthologues.

ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 3(1): 8-13 (2008)

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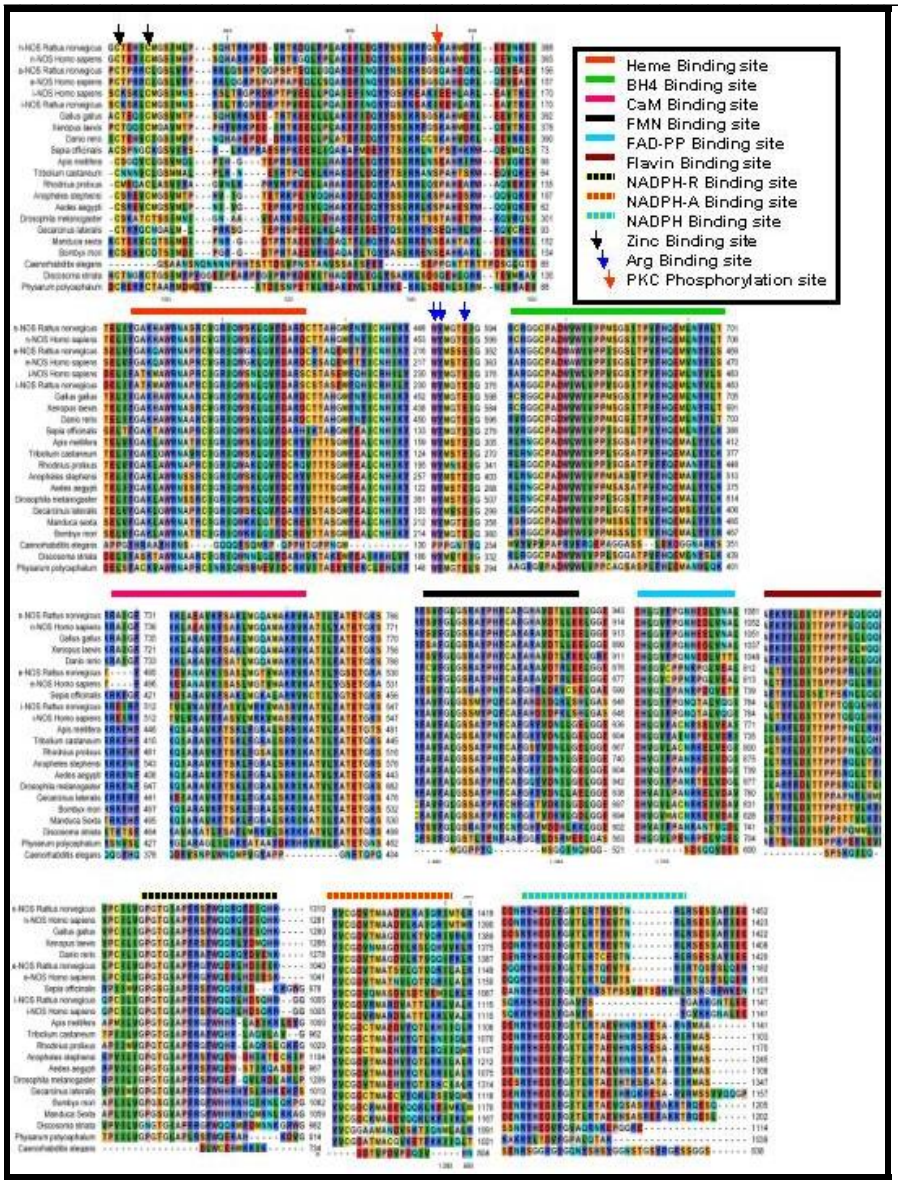

Figure 3: Multiple sequences alignment (MSA) of different Nitric Oxide Synthase (NOS) homologues from insect and mammalian origin using Clustal W Program. Amino acids are represented as single letter i.e. A-Ala, C-Cys, D-Asp, E-Glu, F-Phe, G- Gly, H-His, I-Ile, K-Lys, L-Leu, M- Met, N- Asn, P-Pro, Q-Gln, R-Arg, S-Ser, T-Thr, V-Val, W-Trp and Y-Tyr. Conserved PKA phosphorylation site is indicated using red arrow. Binding sites for various cofactors (Heme, CaM, BH4, FMN, FAD-PP, Flavin, NADPH- Ribose and NADPH has been indicated by domain-specific sequence homology. Amino acid sequence for various NOS homologues has been represented in RASMOL. Gap scored between the aligned sequences has been represented as dashed line, introduced for the best alignment.

The tertiary structure prediction of the Dipteran and Lepidopteran NOS against the crystal structure data available for rat neuronal NOS oxygenase (PDB: 1p6kB, X-ray resolution 1.78 Å) and NOS reductase (PDB: 1tllA, X-ray resolution 2.30 Å) domains indicated remarkable similarity between insect and mammalian NOS (Figure 2). The NOS oxygenase domain is formed by one continuous fold made up of several overlapping or winged β-sheets, a feature likely shared between insects and mammals. It was more conserved than the reductase domain as represented by Bombyx NOS_{oxy} (Sequence identity 63%, modeled residue 79-482, E-Value $2.43e^{-162}$) and Drosophila NOS_{oxy} (Sequence identity 62%, modeled residue 217-630, E-Value $9.19e^{-164}$). Reductase domain of Bombyx (Sequence identity 40%, modeled residue 516-1194, E-Value $6.5e^{-144}$) and

ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 3(1): 8-13 (2008)

Drosophila (Sequence identity 42%, modeled residue 666-1341, E-Value $9.14e^{-151}$) has relatively less homology with mammalian NOS_{red}. In oxygenase domain the Zinc binding site (important for dimerization) as well as arginine binding (substrate binding) site are conserved in the three dimensional folded structure of NOS, which indicates analogy in the dimerization and substrate binding activity.

As evident from these results, a highly conserved tertiary structure as well as active domains and sites predict an analogous substrate specificity and catalytic mechanism of insect NOS compared with the mammalian nNOS.

Lepidopteran NOS orthologues predict significant differences in post-translational modification and sub-cellular localization from Diptera and mammalia

When NOS undergoes serine/threonine or tyrosine phosphorylation within its oxygenase domain, this lowers its activity almost two third by an unknown mechanism, possibly due to conformational change that might lead to decrease in cofactor or substrate accessibility. Therefore, phosphorylation sites in the oxygenase domain of Lepidopteran, Dipteran and mammalian NOS were studied using Net Phos 2.0. The predicted phosphorylation sites were conserved between insect and mammalian nNOS with some positive substitution in which serine was replaced by threonine. However, the Lepidopteran NOS had significantly higher phosphorylation score (0.885) as compared to *Drosophila* (0.542) and mammalian (0.694) NOS, which indicates increased post-translational phosphorylation in these insect orthologues (Table 1 in supplementary material). Incidentally, the sub-cellular compartmentalization is also influenced by specific posttranslational phosphorylations of NOS **[6]**. In response to activators or agonists, NOS gets phosphorylated and acquires higher solubility than the unphosphorylated protein, favouring a primarily cytosolic localization. In fact, when NOS orthologues were studied for their sub-cellular localization using WoLFPSORT, the Lepidopteran insect NOS had predominant signal for cytoplasmic localization, whereas most other NOS orthologues showed signals for cytoplasmic and nuclear localization as well as for nucleo-cytoplasmic shuttling (Table 2 under supplementary material).

The primarily cytoplasmic localization (aided by both the cytoplasmic signal and the higher phosphorylation scores) could possibly impart higher resistance to these cells following oxidative stress. In particular, the nitric oxide produced by NOS activity may interact with other ROS like superoxide radicals and can lead to formation of highly deleterious peroxynitrite radicals, which can induce significant nuclear or mitochondrial DNA damage **[17]**. The preferential exclusion of NOS from Lepidopteran cell nuclei and mitochondria can result in reduced proximity of this enzyme with genomic and mitochondrial DNA, which could finally result in the reduced amplification of oxidative damage by external agents.

Phosphorylation of NOS also decreases the positive charge of the region that may additionally contribute to its electrostatic binding of NOS to lipids and enhance membrane localization **[6]**. In addition to cytoplasmic signal, the Lepidopteran NOS also shows a positive but relatively less favoured localization at cell-cell junctions. On the other hand, NOS of higher vertebrates has signals for localization on both plasma membrane and cell-cell junctions. These subtle differences in the localization of Lepidopteran NOS could additionally contribute to differences in the stress resistance.

Conclusion:

The present *in silico* analysis demonstrates that Lepidopteran NOS, while carrying significant similarity with mammalian nNOS, has important structural and functional features that may have strong implications for resistance to radiation and other stress agents. Specifically, the higher phosphorylation scores of Lepidopteran NOS at the well conserved phosphorylation sites may reduce NOS activation in the event of extraneous oxidative stress. Further, the primarily cytoplasmic localization of Lepidopteran NOS, aided by higher phosphorylation scores as well as cytoplasmic localizing signals, can impart significant reduction in the amplification of oxidative insult. Since the sites and extent of post-translational phosphorylation as well as the sub-cellular localization of NOS can effectively modulate stress sensitivity **[18]**, we hypothesize that a higher phosphorylation score coupled with the largely cytoplasmic localization of NOS could significantly contribute towards the radioresistance of Lepidopteran insects as well as their cultured cell lines.

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ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 3(1): 8-13 (2008)

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Edited by P. Kangueane

Citation: Suman *et al.*, Bioinformation 3(1): 8-13 (2008)

Supplementary material

Bombyx mori **(Lepidoptera)**	***Drosophila*** **melanogaster (Diptera)**	**Human (Mammalia)**
103 VCQT**S**IMDI (0.986)	250 TCTS**S**IMNI (0.052)	339 ICMG**S**IMHP (0.006)
134 QYYA**S**IKRE (0.922)	281 QYFT**S**IKRT (0.923)	373 QYYS**S**IKRF (0.886)
140 KREN**S**EAHK (0.903)	287 KRTS**S**TAHE (0.968)	379 KRFG**S**KAHM (0.765)
214 NHIK**Y**ATNK (0.699)	361 NHIK**Y**ATNK (0.699)	453 NHYK**Y**ATNK (0.786)
413 RDNV**S**IVDH (0.896)	560 SRNV**T**IVDH (0.447)	652 SDKV**T**IVDH (0.079)
419 VDHH**S**ASEQ (0.994)	566 VDHH**T**ASES (0.110)	658 VDHH**S**ATES (0.992)
421 HHSA**S**EQFQ (0.769)	568 HHTA**S**ESFM (0.665)	660 HHSA**T**ESFI (0.011)
450 VPPM**S**SSLT (0.888)	597 VPPL**S**GSIT (0.952)	689 VPPM**S**GSIT (0.963)
469 YRPS**Y**DYQ (0.968)	616 YLKP**S**FEYQ (0.906)	708 RLTP**S**FEYQ (0.542)
472 PSYD**Y**QEPP (0.879)	619 PSFE**Y**QDPA (0.958)	711 PSFE**Y**QPDP (0.296)
529 YATE**T**GKSE (0.946)	679 YATE**T**GKSE (0.946)	768 YATE**T**GKSQ (0.793)
535 KSEH**Y**AKEL (0.772)	685 KSEQ**Y**AKQL (0.708)	774 KSQA**Y**AKTL (0.441)
Average P Score: 0.885±0.02	Average P Score: 0.546±0.107	Average P Score: 0.694±0.094

Table 1: Predicted phosphorylation sites in the NOS oxygenase domains of Dipteran (Drosophila), lepidopteran (Bombyx) and Mammalian (Human) NOS. Analogous phosphorylation sites and phosphorylation scores (parentheses) are indicated. Amino acid prone to phosphorylation is indicated in bold alphabet.

Organisms	**Cyto**	**Cyto_ Nucl**	**Nucl**	**Cyto_ mito**	**Mito**	**Cyto_ Plas**	**Cysk**	**Cyto_ Pero**	**Pero**
Physarum polycephalum (Protozoa)	**14.5**	NSF	**14**	NSF	NSF		NSF	NSF	NSF
Discosoma striata (Coelentrata)	**11**	**14.3**	**16.5**	**6.3**	NSF	NSF	NSF	NSF	NSF
Caenorhabditis elegance (Nematoda)	**3**	**15.5**	**26**	NSF	NSF	NSF	NSF	NSF	NSF
Gecarcinus lateralis (Arthropoda; Crustacea)	**9**	**15**	**19**	NSF	NSF	NSF	NSF	NSF	NSF
Apis mellifera (Arthropoda;Hymenoptera)	**10**	**18**	**18**	NSF	NSF	NSF	NSF	NSF	NSF
Tribolium Castaneum (Arthropoda; Coleoptera)	**8.5**	**10.5**	**9.5**	NSF	**10**	NSF	NSF	NSF	NSF
Rhodinus prolixus (Artropoda; Hemiptera)	**8**	**8.2**	**6.5**	NSF	**5.5**	NSF	NSF	**8.3**	**3.5**
Drosophila melanogaster (Arthropoda; Diptera)	**11**	**13.5**	**14**	NSF	**6**	NSF	NSF	NSF	NSF
Bombyx mori (Artropoda; Lepidoptera)	**23**	NSF	**3**	NSF	NSF	NSF	**3**	NSF	NSF
Sepia Officinalis (Mollusca)	**11.5**	**16**	**19.5**	NSF	NSF	NSF	NSF	NSF	NSF
Danio rerio (Vertebrate: Pisces)	**14**	NSF	**13**	NSF	NSF	NSF	**3**	NSF	NSF
Xenopus laevis (Vertebrate; Amphibia)	**6.8**	**12.8**	**17**	NSF	**4.7**	NSF	**4**	NSF	NSF
Gallus gallus (Vertebrate; Aves)	**11**	**13.3**	**13.5**	NSF	**7**	NSF	**3**	NSF	NSF
Rattus Novarticus (Vertebrate; mammal)	**10**	**12.8**	**14.5**	NSF	**6.3**	NSF	**3**	NSF	NSF

Table 2: Predicted cellular localization for NOS homologue derived from various species using WoLFPSORT server. Scores for possible localization sites are indicated in the table. Signals were analysed for Cyto (Cytoplasmic), Cyto_Nucl (Cytoplasmic Nuclear), Nucl (Nuclear), Cyto_mito (Cytoplasmic mitochondrial), mito (mitochondrial), Cyto_plas (Plasmamembrane localization upon Ca++ binding), Cysk (Present on cell-cell junction), Cyto_per (Cytoplasmic peroxysomal) and pero (peroxysomal) localization. NSF means 'no signal found' for the respective site indicated in the table.

ISSN 0973-2063 (online) 0973-8894 (print)
Bioinformation 3(1): 8-13 (2008)