Hypothesis

In search of the prototype of nitric oxide synthase

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Abstract **Recent identification of the prokaryotic genes related to the catalytic oxygenase domain of mammalian nitric oxide synthase (NOS) has led to speculations on the origins of the NO signaling network. NOS activity in eukaryotes relies on the concerted action of the oxygenase domain with an electron-donating reductase domain that is fused to it. A fused reductase domain is, however, absent in prokaryotes. Consequently, we searched bacterial genomes for homologs of the reductase domain and identified candidate genes. On the basis of genomic sequence and protein structural analysis, we here propose that sulfite reductase flavoprotein is a prototype of the mammalian NOS reductase domain and a complementing interaction partner of the bacterial NOS oxygenase protein.**

Key words: Nitric oxide synthase; Nitric oxide signaling; Evolution; Reductase domain; Sulfite reductase

1. Introduction

Nitric oxide is an important signaling molecule that is generated by the family of nitric oxide synthase (NOS) enzymes [1]. Three highly homologous mammalian isoforms of NOS have been identified, namely: neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). All three are homodimers in their physiological states. Each of them is composed of oxygenase and reductase domains [2–7] linked by an inter-domain peptide that binds calmodulin [5,7]. The N-terminal oxygenase domain (NOSoxy) contains binding sites for heme, the substrate L-Arg and the cofactor tetrahydrobiopterin (BH_4), whereas the C-terminal reductase domain (NOSred) has binding sites for reduced nicotinamide adenine dinucleotide phosphate (NADPH), flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) [8–10]. The complex multi-domain structure of different higher eukaryotic NOSs is the result of several gene fusions.

There have recently been a number of reports on the identification of bacterial homologs of the mammalian NOSoxy domain [11–13]. These proteins are shorter in their N-terminal part and therefore lack the zinc ion-binding cysteines, part of the BH_4-binding sites and residues involved in binding the adjacent subunit in the dimer. The crystal structures of bacterial NOS oxygenase domain precursor (bNOSoxy) proteins from *Staphylococcus aureus*, *Bacillus subtilis* and *Deinococcus radiodurans* [14–16] reveal a similar overall fold to mammalian NOS and, despite the missing N-terminal part, the proteins form dimers. bNOSoxy proteins from *D. radiodurans*, *B. subtilis*, *Nocardia*, and *S. aureus* have been shown to have NOS activity, as evidenced by detection of NO/nitrate and citrulline production in the presence of L-Arg and BH_4 [16–19].

BH_4 is commonly known to be absent in prokaryotes [16,20]. However, another reduced pteridine produced by bacteria, tetrahydrofolate, could support NO production from *B. subtilis* and *D. radiodurans* [16,17]. Interestingly, it has been proposed that NO generated by pathogenic organisms could have a critical role during infections [19].

On the other hand, NOS activity in eukaryotes relies on the electron-donating reductase domain (NOSred). The electron transfer pathway is from NADPH through FAD and FMN to the heme domain. Provocatively, it has been verified that bNOSoxy are capable of accepting electrons from a mammalian NOS reductase domain under in vitro conditions [16,17]. This observation suggests that bacterial genomes may also hold a homolog of the NOS reductase domain.

Using a combination of bioinformatics tools including genomic search and comparison as well as protein structural analysis, we point to the sulfite reductase flavoprotein (SiR-FP) as being a prototype of a mammalian NOS reductase domain and a complementing interaction partner of the bacterial NOS oxygenase protein.

2. Materials and methods

To search and compile potential bacterial NOS homologs, the two well known large databases located at www.tigr.org and www.ncbi.nih.gov were queried. The SMART tool (smart.embl-heidelberg.de) [21,22] was applied to determine borders of functional domains in sequences that were then aligned using ClustalX v.1.81 [23].

Superimpositions of the structures of a mammalian nNOS reductase domain (rat, pdb: 1F20) and a bacterial SiR (*Escherichia coli*, pdb: 1DDI) were performed using ProSup v.3.0 [24]. The *S. aureus* SiR FMN domain was modeled using the Swiss-Model modeling server [25] with the *Bacillus megaterium* FMN domain (pdb: 1BVY) as a template. Superimposition-based docking of the modeled bacterial FMN domain to bNOSoxy was done manually with Swiss PDB viewer v.3.7 [26] without energetic refinement. The pdb coordinates of the different models are available on request from the authors.

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Abbreviations: NOS, nitric oxide synthase; bNOSoxy, bacterial NOS oxygenase domain precursor; NOSoxy, oxygenase domain of NOS; NOSred, reductase domain of NOS; BH_4, tetrahydrobiopterin

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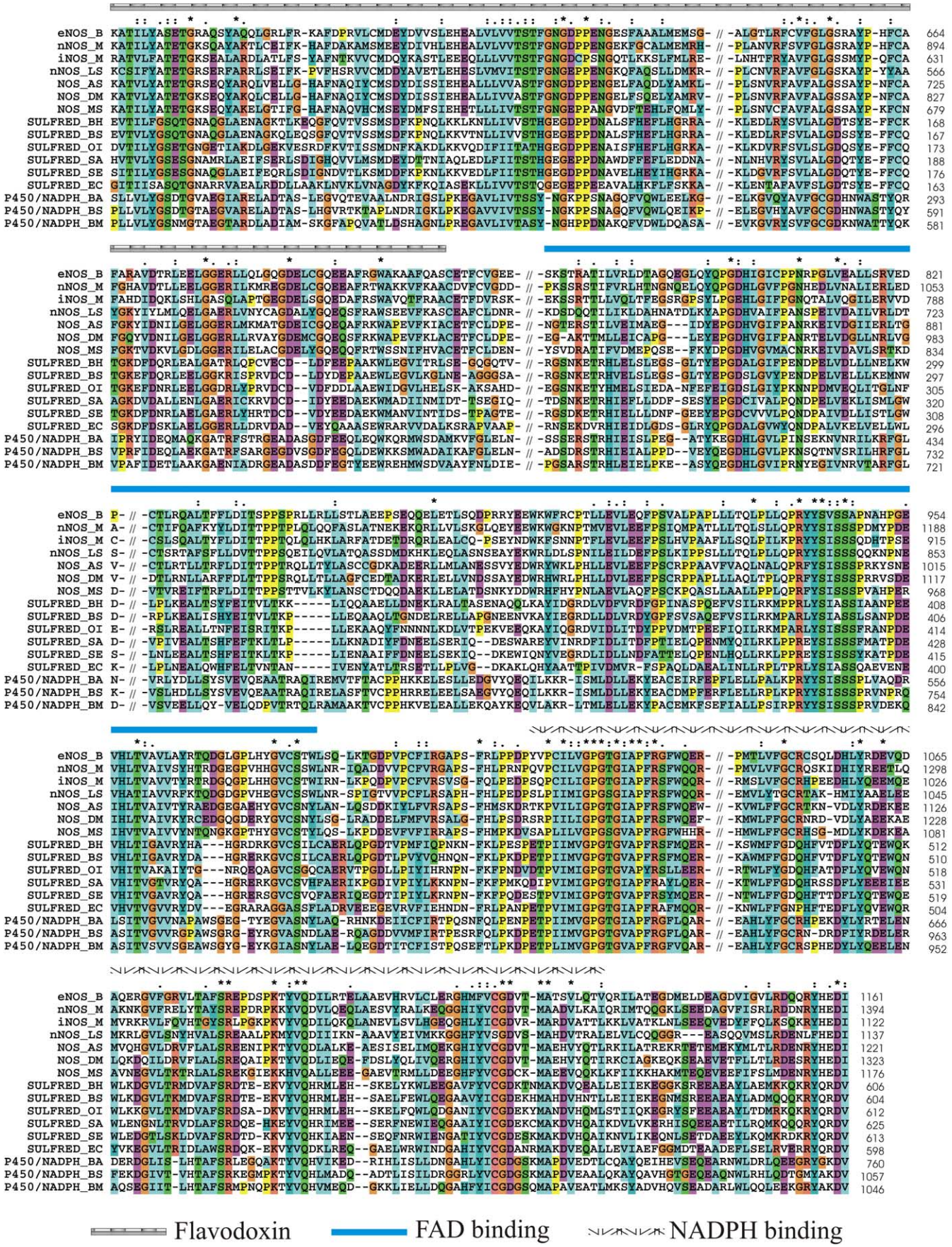
eNOS_B
nNOS_M
iNOS_M
nNOS_LS
NOS_AS
NOS_DM
NOS_MS
SULFRED_BH
SULFRED_BS
SULFRED_OI
SULFRED_SA
SULFRED_SE
SULFRED_EC
P450/NADPH_BA
P450/NADPH_BS
P450/NADPH_BM
Flavodoxin
FAD binding
NADPH binding

Table 1
Occurrence of NOSred homologs in eight bacterial genomes (see text for details)

NOSred homolog	1	2	3	4	5	6	7	8
SiR-FP	+	+	+	+	+	−	−	−
BM-3	+	−	−	−	−	+	+	−

3. Results and discussion

The mammalian NOSred domain possesses the same protein architecture as a family of proteins including SiR, P450 oxidoreductase and cytochrome P450 BM-3, which all contain FMN-, FAD- and NADPH-binding sites. They are all members of the same cluster of orthologous groups (COG), COG0369 (www.ncbi.nlm.nih.gov/COG [27], www.bork.embl.de/STRING [28]).

Given the domain composition of the eukaryotic NOS enzyme, we looked for a prokaryotic reductase domain that, if present, could complement the bNOSoxy protein in bacteria in full functionality.

3.1. Identification of bNOSoxy in bacterial genomes

First, bacterial genomic databases (containing more than 250 genomes) were queried with sequences of oxygenase domains of each of the three mammalian NOS isoforms (neuronal, endothelial, inducible). From this extensive search, we identified only eight bacterial species bearing bNOSoxy. Seven of these are members of the Firmicutes family: (1) *B. subtilis* {168}, O34453; (2) *Bacillus halodurans* {C-125}, NP_241689; (3) *Oceanobacillus iheyensis* {HTE831}, NP_693612; (4) *S. aureus* {COL, N315, Mu50, MW2}, SA1976, NP_374522, BAB58076, BAB95720; (5) *Staphylococcus epidermidis* {ATCC 12228}, AAo05197; (6) *Bacillus anthracis* {A2012}, NP_653913; (7) *Bacillus cereus* {ATCC 14579}, NP_835105, and one of them is a member of the *Deinococcus-Thermus* family: (8) *D. radiodurans* {R1}, Q9RR97.

We repeated the search for bNOSoxy using the identified genes as query sequences but this did not yield any additional homologous sequences.

3.2. Co-existence of bNOSoxy and homologs of the mammalian reductase domain in bacteria

In the next step, we investigated which potential complementing reductase domain, if any, could be identified in these eight bacterial genomes. No protein or domain has been assigned as a NOSred homolog in these bacteria before. We used sequences of the reductase domains from each of the three mammalian NOS isoforms to conduct BLAST searches against the bNOSoxy-containing bacterial genomes. Genomes 1–5 from the Firmicutes family revealed the existence of a highly homologous SiR-FP.

Taking into account the domain architecture and structure, we found only one more protein displaying a domain organization similar to eukaryotic NOSred: a BM-3-like bifunctional cytochrome. It is present in the following three out of the eight bNOSoxy-containing bacterial genomes: *B. subtilis* {168} (CAB12544), *B. anthracis* {A2112} (NP_657092) and *B. cereus* {ATCC 14579} (NP_832952). Table 1 summarizes the occurrence of BM-3 and SiR in bacterial genomes bearing the bNOSoxy.

The alignment in Fig. 1 shows the high conservation of residues between NOSred, SiR-FP and the reductase domain of the BM-3-like cytochrome P450, and allows detailed comparison of the similarity in the domain architecture.

The BM-3-like P450 and the SiR-FP are the only proteins that provide the full functionality of the NOSred domain – that is, the capability of catalyzing electron transfer from the NADPH-binding domain via FAD to the FMN domain. However, structural analysis (data not presented) shows that the reductase domain of BM-3 is already linked with its oxy domain by a peptide and therefore cannot serve as a flexible interaction partner for bNOSoxy.

3.3. Dual functionality of SiR-FP

The SiR-FP identified is known to be an electron-donating partner for SiR hemoprotein (SiR-HP). Both interact strongly in vivo in many bacterial species, catalyzing the transfer of electrons from NADPH to sulfite to produce sulfide. Interestingly, although both SiR-FP and SiR-HP are normally part of one operon in bacterial genomes, the *S. aureus* {COL, N315, Mu50, MW2} genome (#4) lacks SiR-HP, while it has a SiR-FP. This fact argues for SiR-FP also being an interaction partner for other proteins. In Section 3.4, we will examine structural arguments that SiR-FP is capable of donating electrons not only to SiR-HP but also to bNOSoxy protein.

3.4. SiR docked to bNOS can act as an electron-donating system

Whereas the primary structure conservation between NOSred and SiR-FP is suggestive, the question arises as to whether bNOSoxy has a functional docking site capable of interfacing with the reductase domain. This is particularly pertinent as two recent papers describe electron-transfer-active chimeras comprised of bNOSoxy proteins (*D. radiodurans* and *B. subtilis*) and mammalian nNOS reductase domains [16,17].

This is also in agreement with another recent study reporting from purification of heterodimeric NOS from *S. aureus* that the complex contains a reductase domain as a subunit [29].

Interestingly, our structural alignment depicts a high level of homology between the FAD–NADPH domains of mammalian nNOS (rat) and bacterial SiR (*E. coli*) (Fig. 2).

The only reported crystal structure of a complex between the heme domain and the FMN domain is that of the BM-3 cytochrome P450 from *B. megaterium* [30]. It reveals 'cross-

←
Fig. 1. Sequence alignment of reductase domains of SiR (SULFRED_OI: *O. iheyensis* {HTE831}, BAC13609; SULFRED_BH: *B. halodurans* {C-125}, NP_241475; SULFRED_BS: *B. subtilis* {168}, CAB15349; SULFRED_SA: *S. aureus* {MW2}, BAB96405; SULFRED_SE: *S. epidermidis* {ATCC 12228}, NP_765735; SULFRED_EC: *E. coli* {B} P38038), NOS (NOS_MS: *Manduca sexta*, AAC61262; NOS_AS: *Anopheles stephensi*, O61608; NOS_DM: *Drosophila melanogaster*, NP_523541; eNOS_B: endothelial NOS, bovine, P29473; nNOS_M: neuronal NOS, *Mus musculus*, Q9Z0J4; iNOS_M: *M. musculus*, AAM11887; nNOS_LS: neuronal NOS, *Lymnaea stagnalis*, O61309), cytochrome BM-3 (P450/NADPH_BM: *B. megaterium*, P14779; P450/NADPH_BS: *B. subtilis* {168}, CAB12544; P450/NADPH_BA: *B. anthracis* A2012, NP_657092). Three functional domains, FMN, FAD, NADPH, that are conserved among species are outlined just above the sequences in the alignment. Low-homology/non-homologous regions were cut out of the alignment and designated by -//-.

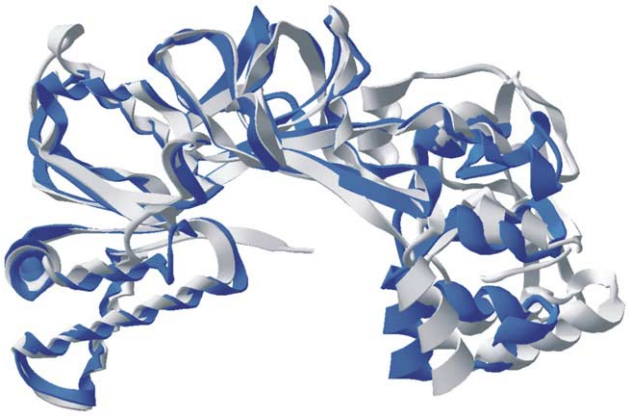

Fig. 2. Superimposition of the FAD/NADPH-binding domain of SiR (*E. coli*, pdb: 1DDI) and the FAD/NADPH-binding domain of nNOS (rat, pdb: 1F20), colored in blue and gray respectively. Root mean square (RMS) value calculated with Prosup v.3.0 (see Section 2) is 1.71 Å.

talk' at the docking interface involving methyl groups of the FMN domain positioned toward the heme-binding loop. Taking into account the homology in the FMN domains of BM-3 P450 and SiRs (Fig. 1), and a similarity in the structures of the BM-3 and bNOSoxy heme-binding loops, one might expect a similar interaction between bNOSoxy and SiR.

We therefore used the P450 BM-3 structure as a template to create a model of *S. aureus* bNOSoxy protein complexed with the FMN domain of the *S. aureus* SiR. The heme-binding loops as well as the heme groups were superimposed for this purpose. In the resulting model, the FMN domain fits well in the cleft present in the vicinity of the NOSoxy N-terminal region (Fig. 3). The model therefore constitutes a possible configuration of a docking mode for bacterial NOSoxy that would not be possible for eukaryotic NOSs with an extended N-terminal part. The mammalian NOSoxy domains of the three known isoforms differ from each other in the peptide length involved in the dimer interface N-terminal extension. Of these, only nNOS is expressed as a catalytically active N-terminally truncated form (nNOSγ) resembling that of bNOSoxy. It has just recently been shown that an N-terminal deletion mutant Δ349nNOS (similar to nNOSγ and bNOSoxy) exhibits a slower heme reduction, possibly due to a changed FMN domain orientation [31]. Therefore, it is tempting to speculate that the docking site for the FMN domain may be different between bacteria and full-length eukaryotic NOSs. This would also be consistent with the recent study of chimeric proteins by Roman et al. [32] that suggests differences in the alignment of the nNOS and cytochrome P450 reductase FMN domains with the NOSoxy domain.

The green arrow in Fig. 3 marks the C-terminal part of the FMN domain (residues colored in yellow), which would be connected with the FAD domain. Consistent with this, it is surface-exposed in the model. We do not propose a single relative orientation of the FMN and FAD/NADPH subunits, due to a fact that the SiR-FP flavodoxin domain is known to sample a wide range of configurations for optimal electron transfer [33]. SiR-FP is octameric in solution [34], and therefore it has been suggested that the FMN domain occupies a central position for the electron transfer to occur [33]. Nevertheless, since the docked structure obtained for bNOSoxy–(SiR-FP)–FMN shows a similar distance between the heme-binding loop and the FMN domain to that of BM-3oxy-FMN, we conclude that this particular domain arrangement allows for efficient electron transfer.

3.5. Observed diversity

We observe diversity among bacterial genomes bearing bNOSoxy manifested in the lack of SiR-FP in three out of eight of them. Among those genomes, *D. radiodurans* contains no reductase domain homolog. The enzymatic function of bNOSoxy could vary among bacterial species depending on environmental pressures. Similarly, the lack of SiR-HP in the *S. aureus* genome (see above) supports SiR-FP being involved in interactions with other partners (such as bNOSoxy). This has also been demonstrated on a functional level using an N-terminally truncated form of SiR-FP, which was capable of transferring electrons to a cytochrome P450 (12–15 times less efficiently than bovine P450 reductase) [35].

3.6. Triggering evolution: from the fusion event to a complex signaling system

The architecture of mammalian NOS, hypothesized here to result from fusion between bNOSoxy and SiR-FP (Fig. 4),

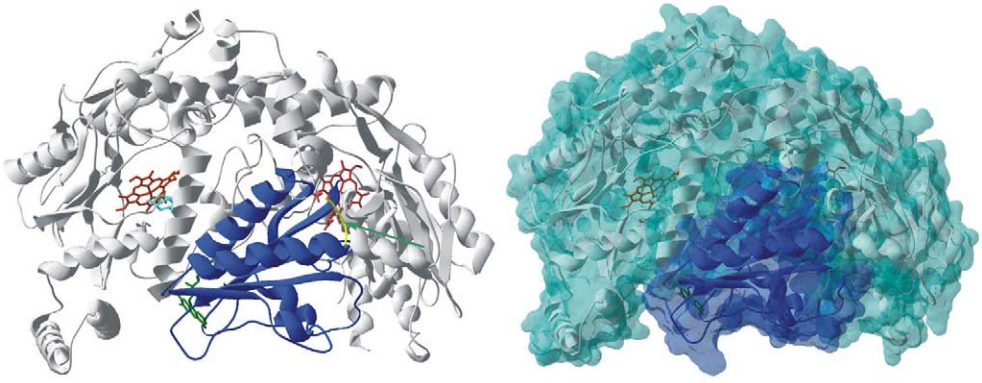

Fig. 3. Docking of the modeled *S. aureus* FMN domain to the bNOSoxy protein (*S. aureus*, pdb: 1MJT) obtained by superimposition on the complex of the heme- and FMN-binding domains of cytochrome P450 (BM-3) (*B. megaterium*, pdb: 1BVY). Dark blue colored ribbons designate the FMN domain. Left: Hemes are in red, the FMN in green, residues in the heme-binding loop used for superimposition in cyan and C-terminal residues in yellow. Green arrow as described in the text marks the C-terminal residues. Right: Molecular surface mapped onto the bNOSoxy and the FMN domains.

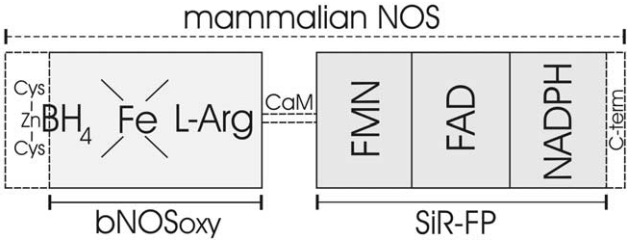

Fig. 4. Schematic representation of the mammalian NOS architecture and its relation to a hypothesized fusion of bNOSoxy and SiR-FP proteins. The dotted regions designate the N-terminal, the calmodulin-binding residues and the C-terminal 20–42-residue regulatory insert acquired by mammalian NOS during the course of evolution.

could only originate from a bacterial genome that contains both proteins (such as the five we identify here).

The result of an envisioned fusion event leading to two domains being expressed in one amino acid chain should allow for efficient NO catalysis in eukaryotes. A similar type of optimization of enzyme turnover efficiency, based on electron flow between two peptide-connected domains, is observed in the bacterial bidomain cytochrome BM-3 system.

The presence of BM-3/NADPH in the three bacterial genomes bearing bNOSoxy suggests (Table 1) that the oxygenase domain of the BM-3-like cytochrome fused with its complementary reductase domain before bNOSoxy did.

We cannot exclude a scenario according to which bNOSoxy does not result in NO-producing function in bacteria. The NO/nitrate-producing actions of a bNOSoxy enzyme would depend on the availability of an electron-donating partner.

The lack of an already fused bNOSoxy–bNOSred system in bacteria (confirmed by an extensive sequence search in over 250 available bacterial genomes) may imply that the introduction of an efficient NO signaling system relying on intensive interactions with an electron-donating reductase domain was only advantageous in the eukaryotic line.

In summary, bacterial SiR-FP, being a close structural homolog of mammalian NOSred (Fig. 2) and a plausible electron-donating partner (Fig. 3), fulfills all the necessary conditions, including detailed structural features, genome co-occurrence and domain architecture, to be a functional complementing partner of the bacterial NOSoxy protein and at the same time emerges as the best candidate identified for a fusion partner giving rise to eukaryotic NOS.

3.7. Outlook

This hypothesis paper should trigger experimental examination of the possibility of electron flow between the FMN domain of SiR-FP and bNOSoxy.

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