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The Evolutionary Origins of Peroxynitrite Signalling

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Abstract

Peroxynitrite is a reactive intermediate formed *in vivo* through uncatalysed reaction of superoxide and nitric oxide radicals. Despite significant interest in detecting peroxynitrite *in vivo* and understanding its production, little attention has been given to the evolutionary origins of peroxynitrite signalling. Herein we focus on two enzymes that are key to the biosynthesis of superoxide and nitric oxide, NADPH oxidase 5 (NOX5) and endothelial nitric oxide synthase (eNOS), respectively. Multiple sequence alignments of both enzymes including homologues from all domains of life, coupled with a phylogenetic analysis of NOX5, suggest eNOS and NOX5 are present in animals as the result of horizontal gene transfer from ancestral cyanobacteria to ancestral eukaryotes. Therefore, biochemical studies from other laboratories on a NOX5 homologue in *Cylindrospermum stagnale* and an eNOS homologue in *Synechococcus* sp. PCC 7335 are likely to be of relevance to human NOX5 and eNOS and to the production of superoxide, nitric oxide and peroxynitrite in humans.

Keywords

peroxynitrite, NADPH oxidase 5, nitric oxide synthase, cyanobacteria, multiple sequence alignment, phylogenetic analysis

Introduction

Peroxynitrite is a pharmacologically important reactive intermediate generated *in vivo* through combination of superoxide and nitric oxide radicals (Fig. 1) [1]. Understanding the medicinal importance of peroxynitrite is challenging, due to its short half-life [2]. We reasoned that a better understanding of the evolutionary origins of peroxynitrite signalling might give insights into the emergence of, and thereby help elucidate, its important biological functions.

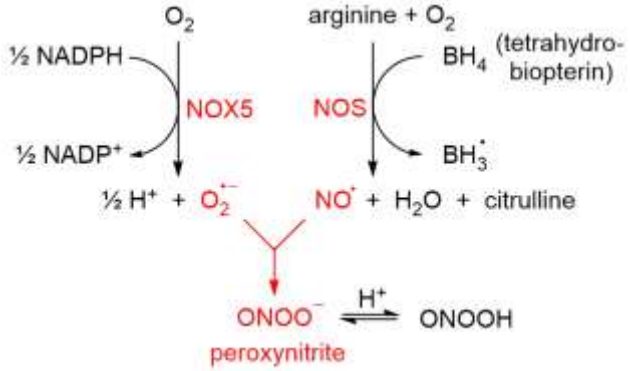


Fig. 1 Production of peroxynitrite *in vivo* requires simultaneous biosynthesis of superoxide $O_2^{\bullet-}$ and nitric oxide $NO^{\bullet}$ by enzymes including NADPH oxidase 5 (NOX5) and nitric oxide synthase (NOS), respectively. The term peroxynitrite encompasses both the peroxynitrite anion and peroxynitrous acid (ONOOH) [3].

The final step of peroxynitrite biosynthesis is uncatalyzed. Therefore, we need to consider the enzymes that produce the two radical precursors (Fig. 1). Nitric oxide (NO) is produced in humans by Nitric Oxide Synthase (NOS), of which there are three main types in mammals, endothelial (eNOS), inducible (iNOS) and neuronal (nNOS) [4]. In humans, the biosynthesis of superoxide is mainly through catalysis by NADPH oxidase (NOX) enzymes, of which one of the subtypes, NADPH Oxidase 5 (NOX5), is believed to be the oldest in evolutionary terms [5].

Peroxynitrite and the enzymes NOS and NOX5 are commonly presented as distinctive features of animal biochemistry. However, as detailed below, protein orthologues have been characterised in non-animal species and recently an all-kingdom phylogenetic analysis of NOS was published [4]. Hence, in line with our own research on other chemical signalling processes [6-8] and Santolini's approach to NOS phylogeny [4], we selected NOX5 orthologues, where available, from the principal kingdoms of all three domains of life (archaea, bacteria and eukaryotes). Coupling this to a detailed analysis of the phosphorylated C-terminus of eNOS/nNOS illuminates the emergence of peroxynitrite signalling during eukaryogenesis.

Beyond the animal kingdom, there have been reports from the Lamattina group of animal-like NOS enzymes in the alga *Ostreococcus tauri* (Ot) and the cyanobacterium *Synechococcus* PCC 7335 [9, 10]. Known as SyNOS, this latter orthologue has been more fully characterised by Picciano & Crane [11]. OtNOS exhibits the characteristic multi-domain primary structure that is seen in animal NOSs (Fig. 2b). This is also true of SyNOS, though the calmodulin-binding domain is absent (see Glob-NOS in Fig. 2b). Both OtNOS and SyNOS are competent for catalytic production of nitric oxide from L-arginine *in vitro*.

Magnani *et al.* selected CsNOX5 from *Cylindrospermum stagnale* for X-ray crystallographic studies because it has 40% sequence identity with human NOX5 and is sufficiently stable for crystallisation [12]. Magnani *et al.* proposed that *C. stagnale* probably acquired the NOX5 gene by horizontal gene transfer (HGT) from a eukaryote. Our analysis, below, paints a different picture of this HGT event.

Results

Our analysis of nitric oxide synthase draws on a phylogenetic analysis by Santolini [4]. Figure 2 presents a simplified sketch summarising the main features of Santolini's unrooted tree, showing how different clades of NOS have distinct multidomain structures. Santolini's analysis makes clear the bacterial, as opposed to archaeal, origins of NOS. It is intriguing that the Glob-NOS clade includes sequences from both eukaryotic protists and prokaryotic bacteria, mainly cyanobacteria, since the standard model of evolution would not situate protists and cyanobacteria as closely related.

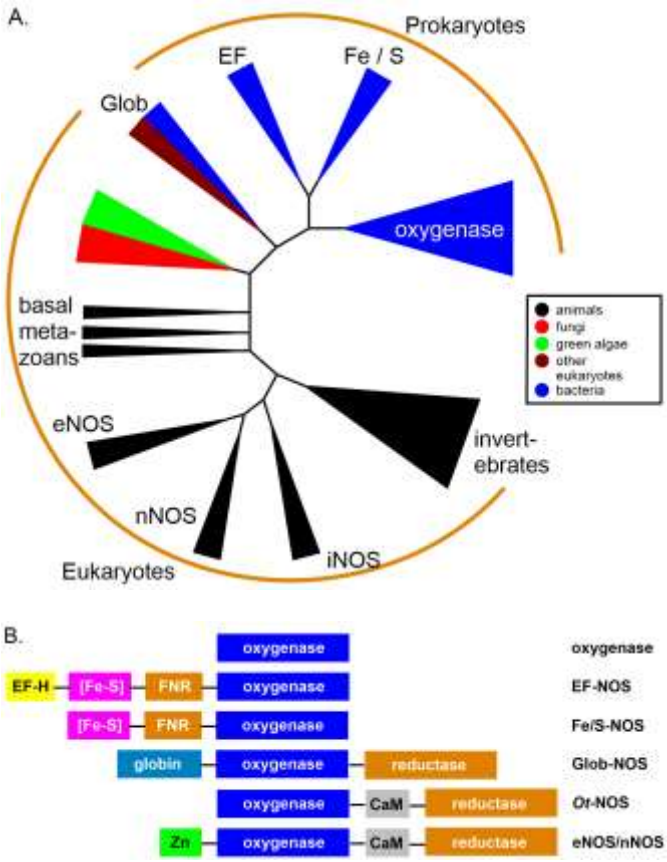


Fig. 2 (a) Simplified sketch of Santolini's phylogenetic tree of nitric oxide synthase homologues [4]; (b) simplified sketch of representative domain structures of NOS homologues in various clades from the phylogenetic tree in (a).

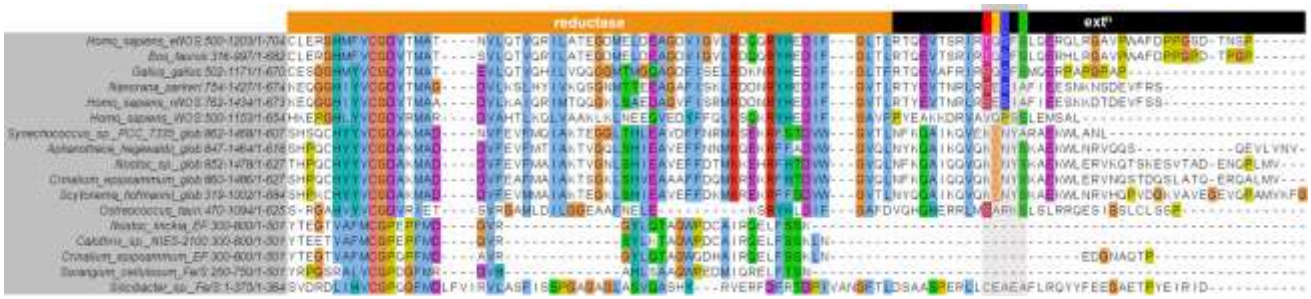
To gain further insight into the provenance of animal NOS enzymes, we looked at a key motif within the sequences. eNOS is the NOS isoform that is usually linked to peroxynitrite production in mammals [13]. However, eNOS and nNOS are reported generally to have overlapping activities [14]. Both eNOS and nNOS have important phosphorylation sites close to the C-terminus of the enzyme and lying beyond the reductase domain [15]. Human eNOS has a motif TQSFS (residues 1175-79), of which the threonine and both serines are known phosphorylation sites. Phosphorylation at serine 1177 is believed to be the most important [16, 17], which aligns with phosphorylated serine 1417 in human nNOS. While there is broad agreement that C-terminal phosphorylation is highly significant to NOS activity, it remains unclear what the biological function is [18].

Using MAFFT [19], we aligned reductase domain sequences from animals and bacterial sequences from the Glob, EF and Fe/S groups identified by Santolini [4], including any C-terminal regions that extend beyond the formal reductase domain. Figure 3a shows clearly that, according to MAFFT, the only bacterial NOS homologues that align at all with the C-terminal extension are from the Glob-NOS group of cyanobacterial proteins plus a single Fe/S group protein from the α-proteobacterium *Silicibacter*.

The TQSFS motif that is phosphorylated in eNOS aligns with a KDNYS motif that is well-conserved across Glob-NOS homologues from cyanobacteria. While the main phosphorylation site, serine 1177, from human eNOS is not found in any of the bacterial sequences in our analysis, the adjacent phosphorylation site serine 1179 is present in Glob-NOS proteins from cyanobacteria, with the exception of *Synechococcus sp. PCC7335*. Further, all the Glob-NOS proteins have a phosphomimetic aspartic acid residue in the position numbered 1176 in human eNOS.

Figure 3b summarises the good alignments between the oxygenase and reductase domains of Glob-NOS and animal NOS, plus the additional match between a short but crucial, in animals at least, C-terminal extension that is characteristic of cyanobacterial Glob-NOS and animal NOS proteins.

A.



B.

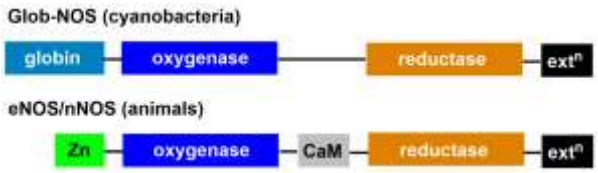


Fig. 3 (a) Detail from the MAFFT alignment of representative NOS homologues, showing the latter section of the reductase domain highlighted in orange and, where present, a C-terminal extension highlighted in black. The 1175-1179 motif that contains phosphorylation sites in human eNOS is highlighted in grey, with serine, threonine and aspartate residues highlighted. (b) Comparison of domain structures in cyanobacterial GlobNOS and animal eNOS/nNOS.

We are aware of only one previous phylogenetic analysis of NOX5 protein sequences [20], which focused mainly on animal NOX5 enzymes. This study included just one bacterial sequence, though it was from a cyanobacterium *Acaryochloris marina*.

We used BLASTp to search the NCBI RefSeq database for potential orthologues of NOX5, using both human NOX5 and *C. stagnale* NOX5 as query protein sequences. Among prokaryotic sequences, we only found close matches among cyanobacteria and γ-proteobacteria. Importantly, we did not uncover potential orthologues from either α-proteobacteria or from archaea. We included the closest BLASTp matches from fungi, from plants and from algae. Finally, we included examples of animal NOX1 and NOX3 protein sequences to act as an "outgroup" in our phylogenetic analysis.

Our database of candidate NOX5 orthologues and paralogues was aligned with MAFFT [19]. The phylogenetic analysis was undertaken in the PhyMl web server using Smart Model Selection (SMS) [21], and fast likelihood-based method aLRT SH-like to generate branch support [22]. The resulting tree is shown through visualisation in TreeGraph 2.0 [23] in Figure 4. The confidence values at all the key Nodes 1-4 are >0.9.

Node 1 represents the last common ancestor of animal NOX1, NOX3 and NOX5 proteins. Our tree shows two clades that have diverged since Node 1. One comprises animal NOX1 and NOX3 proteins, together with sequences from some fungi, plants and algae. The other clade includes animal NOX5 proteins and two groups of candidate bacterial NOX5 orthologues, from γ-proteobacteria and from cyanobacteria.

Node 2 represents the last common ancestor of animal and bacterial NOX5-like sequences. Node 3 is the last common ancestor of animal and cyanobacterial NOX5-like proteins.

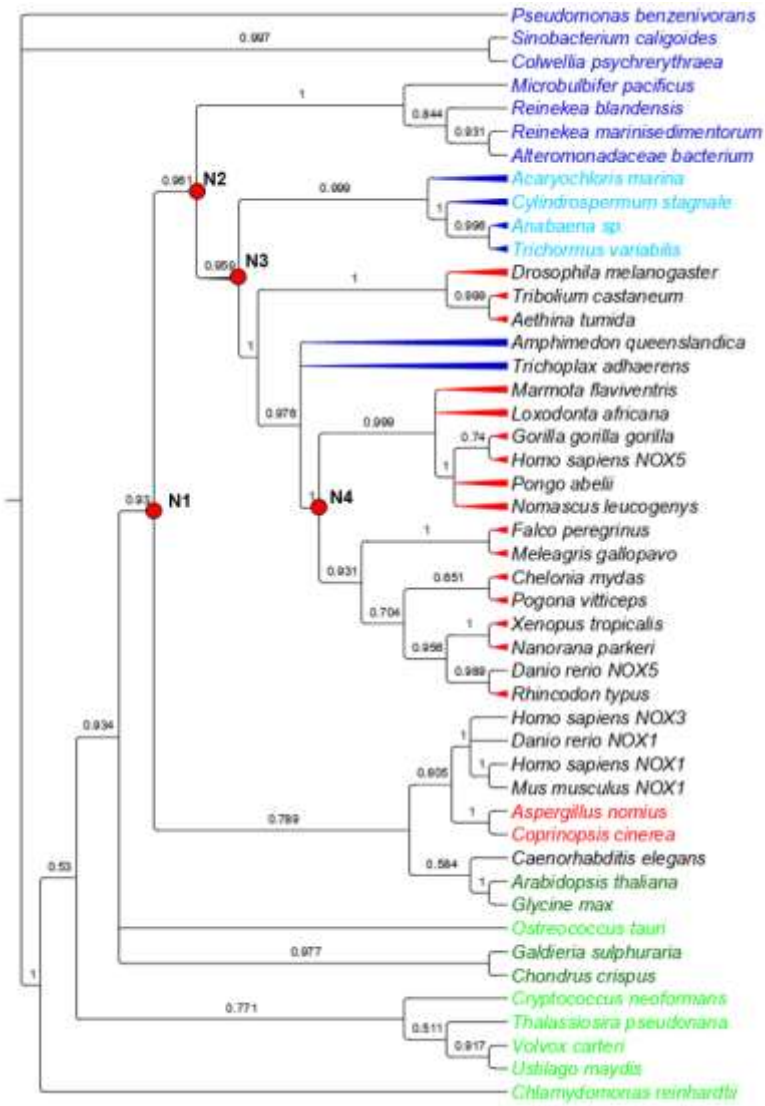


Fig. 4 Phylogenetic tree of NOX5-like protein sequences. Species names are coloured black (animals), red (fungi), dark green (plants), light green (algae), cyan (cyanobacteria) and blue (other bacteria). Branches are coloured blue (Trp378), red (Trp379) or black (no Trp at 378 or 379). N1 to N4 refer to nodes discussed in the text.

Discussion

It seems that animals acquired their NOX5 gene neither from an archaeon nor from an α-proteobacterium, as might be expected from standard endosymbiotic theory. Rather, animals, but not other eukaryotes, appear to have gained NOX5 from an alternative HGT event, between an ancestral cyanobacterium and an ancestral animal, at Node 3 in Figure 4.

In their structural study on *C. stagnale* NOX5 (CsNOX5), Magnani *et al*. identified a key tryptophan residue Trp378 (numbering from *C. stagnale* 5O0X_A) that they propose mediates electron transfer between the two hemes in the active site of the enzyme. The equivalent position in the human orthologue is valine 362, but the same authors note that the adjacent tryptophan 363 may fulfil the same function (this corresponds to position 379 using the *C. stagnale* numbering).

With the exception of plant and algal sequences, most of the NOX and NOX-like proteins in the clades diverging from Node 1 have an aromatic Trp, Phe or Tyr corresponding to CsNOX5 Trp378. There is however a clear demarcation at Node 3. Nearly all the cyanobacterial and animal NOX5 proteins have a tryptophan, either at 378 or 379. The shift from Trp378 to Trp379 is another clear feature of the phylogenetic tree, occurring at Node 4. We should note that the insects in the tree show a somewhat different pattern, with aromatic residues at both the 378 and 379 positions.

Overall, the simplest explanation for the data in Figure 4 is that NOX5-like proteins with a tryptophan in the key electron-transfer position evolved in cyanobacteria and are present in animals thanks to a horizontal gene transfer (HGT) event between an ancestral cyanobacterium and an ancestor of animals. The Trp378 is conserved in basal animals, but shifted to position 379 in "higher" animals.

The most widely accepted model of eukaryogenesis holds that the ancestral eukaryote was formed from an endosymbisosis between an ancestral archaeon and an ancestral α-proteobacterium, though different views are emerging [24]. Therefore, insofar as an animal gene has prokaryotic origins it might be expected to most closely resemble genes from archaea or from α-proteobacteria. Our searches for NOX5-like and NOS reductase domain-like sequences in archaea found no examples. Indeed, Santolini observed that there are very few archaeal examples of NOS-like proteins [4]. It therefore seems certain that NOS and NOX5 originate in the bacterial domain. As mentioned above, Magnani *et al.* proposed that *C. stagnale* had acquired its animal-like NOX5 by HGT from an animal [12]. The presence of NOX5 in a range of diverse cyanobacteria, and the fact that cyanobacteria existed long before animals, suggests the opposite is true. We propose that an ancestral animal acquired the NOX5 gene from an ancestral cyanobacterium.

Hagemann's group have argued, in a study of glycolate oxidase and related enzymes, that "a simple and parsimonious explanation for the unexpected cyanobacterial protein origins is an early HGT between an ancestral cyanobacterium and the common ancestor of eukaryotes" [25]. Likewise, and consistent with our own earlier publications, it seems that all eukaryotes, and in particular animals, have acquired their NOX5 gene from an ancestral cyanobacterium. The phylogenetic proximity of Glob-NOS and animal NOS, the striking similarities in their domain structures, including the important C-terminal extension, and similarities in key biochemical motifs needed for peroxynitrite biosynthesis also point to a close evolutionary link.

Conclusion

The proposed HGT of the two key enzymes NOS and NOX5 that catalyse the biosynthesis of nitric oxide and of superoxide, and hence peroxynitrite, from ancestral cyanobacteria to ancestral animals does not sit easily with the standard model of eukaryogenesis, but accords with a growing body of evidence from ourselves and others that cyanobacteria have made important contributions to the animal genome.

Our findings suggest that the experimental work on cyanobacterial NOS and NOX5 proteins from the groups of Lamattina, Picciano & Crane and Magnani is of heightened relevance to our understanding of peroxynitrite biosynthesis in animals. When proteins are closely related in evolutionary terms, it is likely that their biochemistries will also be similar, even if their biological functions are different. Further computational and experimental studies on cyanobacteria should reveal new aspects of the provenance and biological role of peroxynitrite.

Acknowledgements

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Materials and Methods

NOX5 and related sequences were chosen from NCBI BLAST searchers, using human NOX5 (Uniprot Q96PH1, NCBI NP_078781) and CsNOX5 from Ref. 12 (NCBI WP_015206836) as query sequences. Amino acid sequences were aligned using MAFFT [19]. Phylogeny was inferred using Smart Model Selection in PhyML [21]. Full details of NOX5 and homologous sequences are in Supplementary Table 1. Annotation was undertaken in Treegraph [23].

NOS homologues were retrieved from NCBI to be representative of the key clades described in Ref. 4. The sequences were aligned using MAFFT [19] before visualisation and analysis in Jalview (jalview.org). Full details of the NOS homologues are in Supplementary Table 2.

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