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The NOS-like protein from the microalgae *Ostreococcus tauri* is a genuine and ultrafast NO-producing enzyme

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ABSTRACT

The exponential increase of genomes' sequencing has revealed the presence of NO-Synthases (NOS) throughout the tree of life, uncovering an extraordinary diversity of genetic structure and biological functions. Although NO has been shown to be a crucial mediator in plant physiology, NOS sequences seem present solely in green algae genomes, with a first identification in the picoplankton species *Ostreococcus tauri*. There is no rationale so far to account for the presence of NOS in this early-diverging branch of the green lineage and its absence in land plants. To address the biological function of algae NOS, we cloned, expressed and characterized the NOS oxygenase domain from *Ostreococcus tauri* (OtNOSoxy). We launched a phylogenetic and structural analysis of algae NOS, and achieved a 3D model of OtNOSoxy by homology modeling. We used a combination of various *spectroscopies* to characterize the structural and electronic fingerprints of some OtNOSoxy reaction intermediates. The analysis of OtNOSoxy catalytic activity and kinetic efficiency was achieved by stoichiometric stopped-flow. Our results highlight the conserved and particular features of OtNOSoxy structure that might explain its ultrafast NO-producing capacity. This integrative Structure-Catalysis-Function approach could be extended to the whole NOS superfamily and used for predicting potential biological activity for any new NOS.

1. Introduction

Nitrogen monoxide (NO) is an extremely reactive radical molecule that has long been considered as a toxic, polluting and harmful gas [1]. A few decades ago, the biological history of NO unfolded thanks to the discovery of its central role in the regulation of mammalian vascular tone. The biological source of NO was identified soon after as being three different isoforms of a family of enzymes, named NO-Synthases (NOS). Despite significant differences (histological and subcellular localization, expression profiles, catalytic regulation [2–4]) these isoforms were found to share the same quaternary organization, the same crystallographic structure of their catalytic site and the same catalytic mechanism. If NO has become a ubiquitous signaling molecule regulating many physiological processes such as neural communication, cell cycle, metabolism [5], a considerable part of NOS/NO biochemical history remains related to the cytotoxic reactivity of NO and the utilization of NO and Reactive Nitrogen Species (RNS) in the non-specific immune response [6]. Today, because of the complexity of NO biological chemistry, it is becoming more and more difficult to account for the multiple functions of NOS and NO (from signaling to oxidative stress) and to understand the paradox of NOSs that are at the same time the support of essential physiological functions and the source of numerous pathological conditions [7].

The presence of hundreds of NOS throughout the Tree of Life (personal data) has added another layer of complexity. Indeed, NOSs have been found in all kingdoms of life, in archaea, bacteria, fungi, insects, crustacean, fishes… [8–12]. As the predominant paradigm of the NO field is mostly based on the relation "NOS → NO → Signaling", the newly discovered NOS were *a priori* assigned the same catalytic functioning and the same biological function, namely producing NO

Abbreviations: Arg, L-arginine; BH_4, tetrahydrobiopterin, (6R)-5,6,7,8-tetrahydro-L-biopterin; CO, carbon monoxide; NO, nitric oxide; NOHA, N^{ω}-hydroxy-L-arginine; NOS, nitric oxide synthase; bacNOS, bacterial nitric oxide synthase; bsNOS, nitric oxide synthase from *Bacillus subtilis*; eNOS, bovine endothelial nitric oxide synthase; iNOS, murine inducible nitric oxide synthase; mNOS, mammalian nitric oxide synthase; nNOS, rat neuronal nitric oxide synthase; OtNOS, nitric oxide synthase from *Ostreococcus tauri*; NOSoxy, oxygenase domain of NOS; RNS, Reactive Nitrogen Species; ROS, Reactive Oxygen Species

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and mediating signals [5]. Though, these NOSs have been poorly characterized in comparison with their mammalian counterparts and only a few NOSs from drosophila [13,14], amoeba [10,15] and bacteria [16–24] have been investigated.

Since the late 90s, NO has emerged as a major signaling molecule in plants [25], involved in processes as diverse as plant immunity [26], systemic acquired resistance [27], growth and development [28], N_2-fixing symbiosis tuning [29], or abiotic stresses response [30]. If NO was shown to interact with other signaling molecules, including ROS, Ca^{2+} and hormones such as auxin, salicylic acid and abscisic acid, the precise ways of its biological activity remain to be established [26]. The source of NO production has been and remains also a matter of debate in plants [31,32]. Because of the central role of NO, the discovery of a NOS-like protein in *Arabidopsis thaliana* was proposed [33–35] but rapidly questioned. Indeed, the analysis of the genome of *A. thaliana* and later of those of other higher plants led to the surprising conclusion that there were no mammalian-like NOSs in plants. Today the actual source of NO, the nature of the substrate (Arg, nitrite, nitrate, polyamines) and of the triggered signal (Ca^{2+}, ROS, cGMP) still remains to be determined [36–40].

In this very context, the laboratory of Lamattina (Mar del Plata University, Argentina) spotted in 2010 the presence of a NOS-related sequence [9] in the genome of the microalgae *Ostreococcus tauri* [41]. Ostreococcus belongs to the class of the mammielophyceae,[1] an early-diverging branch (at least 700 million years ago) from the green plant lineage. This picophytoplankton is the smallest eukaryote found to date. It bears a small and compacted genome, with only one mitochondria and one chloroplast. Its broad diffusion corresponds to various ecological niches from coastal to oligotrophic waters, from deep-see to high-irradiance exposure. NOSs have been also detected in other species from this taxon including *Ostreococcus lucimarinus* and *Bathycoccus prasinos* [8]. More recently, using the 1000 Plants (1KP) international multidisciplinary consortium's transcriptome database (www.onekp.com), Wendehenne and colleagues showed that NOSs were to be found in some other species throughout the various classes of algae [42]. NOSs sequence was even found in the fresh-water algae *Chaetosphaeridium globosum*, a member of the Coleochaetophyceae class, a class that is the closest branch to terrestrial plants. However, no NOS sequence has been found in land plants so far, which could be rationalized by at least two hypotheses: (i) NOS sequences were lost during the evolution or (ii) they never have been present. In the first hypothesis, a large range of algae and especially some that have experienced a strong compaction genome would have conserved this enzyme throughout evolution. In the second hypothesis *O. tauri* and the other algae would have gained – from horizontal genome transfer – and conserved the NOS sequences during their evolution as an independent event, perhaps even after land colonization by terrestrial plants. In both cases this questions the reason of the presence of such an enzyme in *O. tauri* and these various algae. Is there any ecological specificity to this organism that required NOS and its beneficial activity? Which biochemical activity NOS is supposed to play in these organisms, and is it related to the catalytic production of NO like in animals?

The characterization of NOS biological activity from these algae is the first necessary step to deal with these questions, with *Ostreococcus* as the best-suited model. The first functional characterization confirmed that NO production in *O. tauri* was OtNOS- and L-Arg dependent and was shown to increase under high light irradiance, suggesting a role of OtNOS in the repair or the defense against photo-induced oxidative damage [9]. More recently, Foresi and colleagues transformed *A. thaliana* plants with OtNOS and observed an increased NO production in relation with a greater resistance to water stress [43]. At this stage, deciphering OtNOS biochemistry constitutes an excellent opportunity to go further into NOS biological chemistry. In this regard, we present here our results on OtNOS structural and functional characterization. Using a combination of spectroscopies and fast kinetics, we comparatively analyzed OtNOS catalytic and kinetic efficiency, its interaction with ligand, substrate and cofactor, and its capacity to produce NO. OtNOS appears as genuine NOS, with similar spectroscopic fingerprints to mammalian NOSs and high NO producing capacity.

2. Experimental procedures

2.1. Bioinformatics and computational

All NOS protein sequences were extracted from NCBI by similarity search from either OtNOS or *Mus musculus* iNOS sequences. For phylogenetic analysis, 39 full-length sequences of NOSs representative of the genetic diversity of this family of proteins were selected (see Fig. 1 legend). Phylogenetic trees were computed through Seaview 4.5 graphical interface using PhyML algorithm, and the figures produced by FigTree.

Jalview 2.8 software was used to generate various multiple sequence alignments. Different algorithms were applied (ClustalW, Muscle, Probcons) and compared. By assessment with the three-dimensional structures of mammal and bacterial NOSs available in PDB, the PROBCONS alignment was considered and further used for phylogeny and homology modeling procedures.

For homology modeling procedure, the set of NOS sequences used for phylogenetic analysis was restrained to a subset of 16 sequences including the 3 algae species, 3 cyanobacteria, 3 mammalian, 3 bacterial, 1 fungal, 1 insect, 1 mollusk, and 1 diatom sequences. This subset was set up for anchoring precisely the NOS oxygenase domains that appeared to be well-conserved in the multiple alignments. The oxygenase domain sequences of algae NOSs were then extracted from the PROBCONS multiple alignment based on the overlap with the well-characterized iNOSoxy domain (segment 127-494 in *Mus musculus* iNOS residue numbering). Among the 16 species of the subset, 5 have been crystallized and structurally determined. This allowed refining the PROBCONS alignment by manual adjustment (see Fig. 2 and S1 in Supplementary material).

The three-dimensional model of *Ostreococcus tauri* NO-synthase oxygenase domain was rebuilt using the homology modeling suite Modeller (9v14) with 1NOD PDB structure as template (chain A). The Cys49-Lys434 domain of OtNOS was rebuilt from the alignment with the segment Cys109-Gln496 of 1NOD chain A structure. The cofactors HEM and BH4, and the substrate ARG have been included in the pairwise alignment, for rebuilding an OtNOS model in the catalytical state. The validated pairwise sequence alignment used as input for Modeller is the same as that displayed in Fig. 2 and S1 in Supplementary material, by extracting lines 4 (*Mus*) and 5 (*Ostreococcus*) from the multiple alignment. Runs of 100 models were performed with a further loop refinement protocol, and the generated models sorted by the MODELLER objective function were evaluated by their DOPE (Discrete Optimized Protein Energy) and GA341 scores calculated by Modeller. The best models corresponding to the lowest DOPE score and best objective function issued from each run were pooled and submitted to the online metaserver SAVES (Structural Analysis and Verification Server: http://services.mbi.ucla.edu/SAVES), and finally to the QMEAN scoring function server (https://swissmodel.expasy.org/qmean) for model quality assessment. The final model was the best one according to a good compromise between the scores calculated by the SAVES server scoring programs and the QMEAN value. As a result, the selected OtNOS structural model had a DOPE score of −41721 and QMEAN score equal to 0.690, which is good when compared to the QMEAN scores of individual iNOS PDB templates. Other mono-template 3D models were rebuilt with various mammal and bacterial structures available in PDB, and the 1NOD template gave the best DOPE and QMEAN scores.

[1] http://www.algaebase.org/search/species/detail/?species_id = T6f1f7ac43489a845.