Architecture of the NADPH oxidase family of enzymes

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A B S T R A C T

The NADPH Oxidases (NOX) catalyze the deliberate production of reactive oxygen species (ROS) and are established regulators of redox-dependent processes across diverse biological settings. Proper management of their activity is controlled through a conserved electron transfer (ET) cascade from cytosolic NADPH substrate through the plasma membrane to extracellular O$_2$. After decades-long investigations of their biological functions, including potential as drug targets, only very recently has atomic-resolution information of NOX enzymes been made available. In this graphical review, we summarize the present structural biology understanding of the NOX enzymes afforded by X-ray crystallography and cryo-electron microscopy. Combined molecular-level insights predominantly informed by DUOX1 full-length Cryo-EM structures suggest a general structural basis for the control of their catalytic activity by intracellular domain-domain stabilization.

1. Introduction

The NADPH Oxidase (NOX) family of enzymes catalyze the reduction of O$_2$ to reactive oxygen species, superoxide or hydrogen peroxide, coupled to the oxidation of NADPH [1–3]. The seven mammalian enzyme isoforms in the NOX Family (NOX1-5, DUOX1-2) are often found uniquely expressed in specific cell types and tissues highlighting their particular roles in specific functional circumstances [4,5], and relevance in disease [6–11]. While much is known about their biological functions, considerably less is understood concerning the structural basis by which their activity is controlled. With the first reports of atomic-resolution structures of NOX5 [12] and DUOX1 enzymes [13, 14], molecular-level information has very recently informed the structural basis for their regulation (Table 1). This structure-focused graphical review presents the current understanding of protein domain architecture and the molecular nature that governs NOX enzyme activity.

2. Overall primary structure

As membrane-associated proteins, all NOX enzymes consist of a cytosolic dehydrogenase (DH) domain and 6-pass-transmembrane heme-coordinating transmembrane (TM, blue in Fig. 1) domain [1, 15]. The DH domain (orange in Fig. 1) comprises an N-terminal lobe that binds the FAD co-factor (FAD Binding Lobe, FBL) and a C-terminal NADPH binding lobe (NBL). The calcium-dependent NOX5 and DUOX1/2 enzymes contain four and two calcium-binding EF-hand (EF) domains, respectively, N-terminal to the TM domain [16]. Both DUOX1/2 contain an N-terminal peroxidase-homology domain (PHD), an additional transmembrane helix (0) and a pleckstrin homology-like domain (PHLD). The activity of the majority of NOX enzymes is initiated by binding of specific proteins, such as p22phox, Rac, and others, whereas NOX4 is the only isoform proposed to be constitutively active as well as negatively regulated by ATP binding [17,18]. Specifically, DUOX1/2 stability and subcellular trafficking is dependent on co-expression and complexation with their corresponding maturation factors DUOXA1/2, [19]; [20] which will be discussed in the context of the DUOX1-DUOXA1 dimer structures (Section 4).

3. NADPH substrate binding to the dehydrogenase (DH) domain

An initial deposited X-ray crystal structure of human NOX2 defined the overall fold of the NADPH binding lobe (NBL) of the DH domain (Fig. 2A). Pioneering efforts by Mattevi and co-workers reported the complete DH domain X-ray crystal structure from \textit{Cylindrospermum stagnale} (cs)NOX5 defining the FBL with bound FAD as well as the NBL containing a C-terminal mutant peptide (red -PWLELAAA in Fig. 2B) [12]. The cNOX5 DH domain NBL fold is practically identical to the NBL of NOX2 (Fig. 2A) as well as the full DH domains characterized in the full-length DUOX1 complexes determined with Cryo-EM (Fig. 2C) [13, 14].

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https://doi.org/10.1016/j.redox.2022.102298
Received 2 March 2022; Received in revised form 16 March 2022; Accepted 16 March 2022
Available online 18 March 2022
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covalent bond with the conserved C668 cysteine (but lack the nicotinamide group due to insufficient electron density points for structure-guided drug design. These collective structures represent efficient starting numbers) [23], these collective structures represent efficient starting points for structure-guided drug design. Given the high degree of conservation of the C-terminal residues, and their apparent flexibility, this segment may play a role as a regulatory toggle switch by influencing the position of NADPH that allows for catalysis. Structural studies by Sun show bound NADPH (PDB: 6WXV), expecting anchored to the NBL with the C-terminal residues above the cellular surface covering the outer surface of the TM domain and makes contacts with the extracellular domains of DUOXA1. However, several questions remain regarding this domain as no hemes are found in these structures. Expectedly, the PHD domain is located at the extracellular surface, however without bound hemes but cation binding sites central to the Fe-atom consistent with an outer-sphere ET process. Both water molecule bound by two histidine residues and an arginine adjacent to the oxygen-reducing center can be identified by a highly ordered (CBS) [13, 14]. Expectedly, the PHD domain is located at the extracellular surface, however without bound hemes but cation binding sites central to the Fe-atom consistent with an outer-sphere ET process. Both water molecule bound by two histidine residues and an arginine adjacent to the oxygen-reducing center can be identified by a highly ordered (CBS) [13, 14].

### Table 1

| NOX Enzyme | Organism     | PDB    | Overall High resolution (Å) | Domains                   | Method         | Notes                          | Citation |
|------------|--------------|--------|-----------------------------|---------------------------|----------------|-------------------------------|----------|
| NOX2       | Homo sapiens | 3AIH   | 2.00                        | DH (NBL) only             | X-ray crystallography | –                             | [12]     |
| NOX5       | Cylindrospermum stagnale | 500X | 2.20                        | DH                        | X-ray crystallography | C-terminal Mutant binds to the NADPH binding side | [12]     |
| NOX5       | Cylindrospermum stagnale | 500T | 2.05                        | TM                        | X-ray crystallography | –                             | [12]     |
| NOX5-CaM   | Mus musculus | 6SZ5   | 2.23                        | –                         | X-ray crystallography | –                             | [16]     |
| DUOX1-DUOXA1 | Mus musculus | 6WXU | 2.70                        | TM, PHD                   | Cryo-EM         | dimer-of-dimer inactive form | [13]     |
| DUOX1      | Mus musculus | 6WXR | 3.20                        | TM PHD DH                 | Cryo-EM         | dimer with bound NADPH active form | [13]     |
| DUOX1      | Mus musculus | 6WXV | 3.30                        | TM PHD DH (NADPH diff)    | Cryo-EM         | Dimer-of-dimer low-calcium inactive form | [14]     |
| DUOX1      | Homo sapiens | 7D3E | 2.80                        | TM PHD DH PHLD EF         | Cryo-EM         | Dimer-of-dimer high-calcium active form | [14]     |
| DUOX1      | Homo sapiens | 7D3F | 2.60                        | TM PHD DH PHLD EF         | Cryo-EM         | –                             | [14]     |

respectively, which are a small transmembrane protein that is contained in DUOX1 cryo-EM structures, it consists of an extracellular domain (ECD) and five transmembrane helices. Black outlines define structural domains and darker shading indicates transmembrane helices or structured lobes in the DH domain. Dashed lines indicate the region of DUOX1/2 that is related in domain architecture with NOX1-5.

![Fig. 1. Primary sequence structure of the NOX enzyme family and DUOXA maturation factors.](image)

The simplest NOX enzymes (NOX1-4) consist of a conserved 6-helix transmembrane (TM) domain that is membrane embedded and an N-terminal dehydrogenase (DH) domain located intracellularly. The DH domain consists of two lobes that bind FAD cofactor (FAD Binding Lobe, FBL) and NADPH substrate (NADPH Binding Lobe, NBL). NOX5 is unique as it contains four N-terminal calcium-binding EF hand domains (EF). The dual oxidases (DUOX1/2) are significantly larger and contain extracellular peroxisome proliferator-activated receptor gamma coactivator 1 (PPARγ coactivator 1) and pleckstrin homology domain (PHLD) as well as two EF-hand domains. DUOX1 and DUOX2 rely on maturation factors DUOXA1 and DUOXA2, respectively.

### 4. Coupling NADPH and O₂ redox through electron transfer in the transmembrane domain

All NOX enzymes comprise a six-pass transmembrane 6-helix domain that complex two b-type hemes responsible for transferring electrons from NADPH through the cell membrane [15,22]. The first structure of the csNOX5 TM domain was solved with X-ray crystallography defining the fold of the transmembrane helices and the Fe heme cofactors [12] (Fig. 3A). Subsequent cryo-EM studies showcased the TM domain as the central focal point in the DUOX1-DUOXA1 complex making interactions with nearly every component of the full-length DUOX1 protein as well as DUOXA1 (Fig. 3B) [13,14]. The TM domain appears virtually identical in all available TM-containing NOX enzyme cryo-EM structures and csNOX5 crystal structure indicating that the TM domain is structurally rigid. The oxygen-reducing center can be identified by a highly ordered water molecule bound by two histidine residues and an arginine adjacent to the Fe-atom consistent with an outer-sphere ET process. Both mouse and human DUOX1 structures contain the PHD at the extracellular surface, however without bound hemes but cation binding sites (CBS) [13,14]. Expectedly, the PHD domain is located at the extracellular surface covering the outer surface of the TM domain and makes contacts with the extracellular domains of DUOX1. However, several questions remain regarding this domain as no hemes are found in these domains, as consistent with previous recombinant proteins studies [24], and their binding sites are defined by cation binding sites (CBS in...
Fig. 2. Substrate binding to the Dehydrogenase Domain (DH) of the NADPH Oxidases. A) human NOX2 NADPH Binding Lobe (NBL) of the DH Domain (PDB ID 3A1F). B) csNOX5 DH domain containing FAD Binding Lobe (FBL) with bound FAD (green) and C-terminal mutant PWLELAAA (red) (PDB ID 5O0X). C) DH domain from human DUOX1 full-length structure with bound NADPH and FAD (green) (PDB ID 7D3E). D) Zoom in of FAD binding side of B). E) Zoom in of FAD and NADPH binding site of C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 3. The transmembrane domain facilitates electron transfer from the dehydrogenase domain to the O₂-reducing center. A) The csNOX5 X-ray crystal structure of the transmembrane (TM) domain showcasing the anchoring of Fe-heme cofactors (yellow spheres) into the plasma membrane in a six-transmembrane α-helix domain (PDB: 5O0T). B) The TM domain in context of the human high calcium (active) full-length DUOX1-DUOXA1 complex structure from Cryo-EM. This structure shows the TM domain as central within DUOX1 and complexed in several domain-domain interactions that enable ET through the plasma membrane. The PHD domain completely covers the extracellular surface of the TM domain and forms protein-protein interactions with the extracellular domain of DUOXA1 (PDB: 7D3F). C) Arrangement of NADPH substrate and flow of electrons (red arrows) from FAD co-factor to Heme 2 followed by electron hopping through F1097 aromatic side chain to Heme 1 and reduction of O₂ at the oxygen-reducing center as defined by a highly ordered water molecule (the red sphere in panel C). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
Fig. 3 B). Functional information from site-directed mutagenesis of residues within the CBS prevents proper hDUOX1-DUOXA1 assembly suggesting a role for PHD in protein stability [14]. Additionally, previous studies have implicated the potential roles of protein-protein intermolecular cysteine disulfides bonds in protein maturation and oligomerization [25], however no such disulfides are observed in the cryo-EM complex structures (Section 5). It seems plausible that the PHD may function to promote hydrogen peroxide production from DUOX1 via superoxide dismutation by delaying diffusion from the TM domain oxygen-reducing center, which has likewise been proposed in the case of the extracellular E-loop of the NOX4 TM domain [26]. Importantly, the hDUOX1 high calcium cryo-EM structure showcases all components in the context of the active state needed to describe the flow of electrons from NADPH to the oxygen-reducing center. Accumulated evidence from the DUOX1-DUOXA1 cryo-EM studies indicate that the DH domain is dynamic, and stabilization of the DH-TM domain interface is critical for activation (Section 5) [13,14]. Therefore, the domain-domain interfacial association of FAD and Heme 2 represents the most important step in the catalytic ET cascade responsible for NOX activity. Additionally, an aromatic amino acid side chain (Phe in DUOX1, Trp in csNOX5) is present in all available TM domains indicating the role of electron hopping to facilitate rapid ET rates across the long intramembrane distance from NADPH to O2 [27].

5. Structure-based regulation of DUOX1: A prototype for understanding the NOX family enzymes

Full-length cryo-EM structures of mouse and human DUOX1-DUOXA1 have allowed for the most comprehensive picture of NOX structure and function, including the association of DUOXA1 maturation factor as well as domains unique to DUOX, including the PHD, two EF-hands (EF), and pleckstrin homology-like domain (PHLD). Purification of mDUOX1-DUOXA1 complexes yields either a 1:1 heterodimer ("dimer" in Fig. 4A) or a 2:2 dimer of heterodimers ("dimer-of-dimers" in Fig. 4B and C). The most significant difference in these structures is the apparent flexibility of the cytosolic DH domain. In the study by Sun, DH domain flexibility depends on the dimer status of mDUOX1-DUOXA1 complex since it is resolvable in the dimer but not dimer-of-dimer (Fig. 4A versus B) [13]. Generally, the more flexible a domain the weaker electron density and local resolution for that domain, often preventing accurate placement of atoms. On the other hand, both active and inactive hDUOX1-DUOXA1 cryo-EM structures by Chen and co-workers are characterized as dimer-of-dimers and the electron density maps allow for modeling of all intracellular domains (Fig. 4C) [14].
Both studies indicate that the cystolic DH domains of DUOX1 are dynamically flexible and can be rigidified through dimerization [13] or binding of calcium [14]. A closer examination of the structural differences between the high and low calcium hDUOX1-DUOX1a complexes reveals the direct impacts of calcium-dependent EF-hand conformation on DH domain stabilization (Fig. 4D). The low calcium structure shows the EF-hands in an extended conformation away from the DH domain (Fig. 4D green), which are brought into contact with the DH domain through a large scale (~40 Å) swing upon calcium binding (Fig. 4D brown). This movement of the EF hand domains additionally induces a tilt in the PHLD toward the TM domain producing new salt bridge interdomain interactions (light blue versus dark blue in Fig. 4D). The influence of these calcium-dependent structural changes straightforwardly aids to rigidify the DH domain and very likely allow for effective ET from FAD to HEME 2 (Fig. 3C) [28]. Importantly, while there are no structural changes to the DH domain in the hDUOX1-DUOX1a complex structures, the low local resolution of the DH domain in the high calcium structure is consistent with a more dynamic DH domain and correspondingly higher resolution in the high calcium structure supports the enhanced domain stability in the high calcium structures [14]. It should be noted that the cellular relevance of DUOX1-DUOX1a dimer-of-dimers is unclear and may be a consequence of producing well-behaved purified and cryo-EM amenable protein complexes. Nevertheless, the collective insights from structural biology indicate that DH domain flexibility is controlled in a structure-based mechanism that enables NOX activity by stabilizing the DH and TM domains.

6. Conclusions

Only recently has information on the structural biology of the NOX enzyme family been made available allowing for the visualization of many critical elements that influence their well-recognized biological functions. A combination of X-ray crystallography and cryo-EM have defined catalytic DH and TM domains enabling for atomic-resolution enzyme family been made available allowing for the visualization of structural stabilization of the DH-TM interface can be applied across the NOX enzyme family as well-established protein-protein interactions are known drivers of activity likely by enabling proper rigidification of otherwise flexible cystolic domains. Despite these broad implications, comprehensive structural information on these enzymes is lacking further motivating structural studies to definitively characterize the molecular mechanism of relevance to each NOX enzyme and their roles in human health and disease.

Declaration of competing interest

DEH and AvdV are listed on US Patent No. 10,143,718, “Covalent inhibitors of Dual Oxidase 1 (DUOX1)”.

Acknowledgements

The authors recognize start-up support from The State University of New York (DEH), NIH grants HL085646 and AG075491 (AvdV), and from Associazione Italiana per la Ricerca sul Cancro (IG19808) (AM).