Nitric Oxide 63 (2017) 61–67

Contents lists available at ScienceDirect

Nitric Oxide

journal homepage: www.elsevier.com/locate/yniox

A perspective on conformational control of electron transfer in nitric oxide synthases

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ARTICLE INFO

Article history:
Received 22 June 2016
Received in revised form
5 September 2016
Accepted 6 September 2016
Available online 9 September 2016

Keywords:
Nitric oxide synthase
Cytochrome P450 reductase
Diflavin oxidoreductase
Fluorescence
Protein dynamics

ABSTRACT

This perspective reviews single molecule and ensemble fluorescence spectroscopy studies of the three tissue specific nitric oxide synthase (NOS) isoenzymes and the related diflavin oxidoreductase cytochrome P450 reductase. The focus is on the role of protein dynamics and the protein conformational landscape and we discuss how recent fluorescence-based studies have helped in illustrating how the nature of the NOS conformational landscape relates to enzyme turnover and catalysis.

1. Introduction

Many experimental and computational techniques have shown the importance of protein conformational change in both cell signalling [1–4] and enzyme catalysis [5–9]. It is now commonly believed that X-ray crystallography-derived structural data, although valuable, is insufficient in describing the function of many proteins. This has led to the notion that the structure-function dogma should be expanded to encompass protein dynamics (structure-dynamics-function relationship) [10]. The dynamic profile of a protein can be thought of as a multidimensional conformational landscape, which comprises of 'hill' and 'valley' features representing high and low energy protein sub-states, respectively (Fig. 1) [10,11]. These population sub-states can be easily perturbed, but also voluntarily controlled, by mutagenesis, temperature, pressure, protein-protein interaction, redox chemistry and ligand/inhibitor binding [10].

The field of protein dynamics is gaining increasing attention, as greater insight into protein function is required, in order to be able to develop target-directed pharmaceuticals [12,13] and to rationally design enzymes for bio-catalytic purposes [14,15]. However, the study of conformational changes associated with enzyme turnover is challenging, as dynamics occur over a broad range of time and distance scales, from sub-Ångstrom localised vibrations (femtoseconds) to large domain reorganisation (seconds) [9,16].

Nitric oxide synthase (NOS) is proposed to make defined conformational changes during catalysis. NOS produces the small molecule nitric oxide (NO), which has a broad range of physiological roles from vasodilation to neurotransmission [17]. The three tissue specific NOS isoenzymes are homodimers that function by transferring electrons, which originate from NADPH, to the catalytic NOS haem porphyrin centre. Each NOS monomer is made up of *i)* a C-terminal reductase domain, comprising discrete FAD/NADP(H) and FMN binding domains, *ii)* an N-terminal oxygenase domain, which contains a tightly bound haem B and a tetrahydrobiopterin (H_4B) molecule and *iii)* a binding site for calmodulin (CaM), which links the reductase and oxygenase domains (Fig. 2).

NOS is believed to shuttle between the so called 'input' and 'output' conformational states, which have different orientations of the reductase and oxygenase domains [18]. These two states are thought to be functionally relevant in 'gating' the precise flux of electrons from NADPH to the catalytic haem centre. In recent years there has been a variety of spectroscopic approaches used to probe NOS conformational change. Much of these data are summarised in recently published review articles on the general biochemistry and

Abbreviations: NOS, nitric oxide synthase; CPR, cytochrome P450 reductase; CaM, calmodulin; MSR, methionine synthase reductase; P450 BM3, cytochrome P450 BM3; FAD, flavin adenosine dinucleotide; FMN, flavin mononucleotide; FRET, Förster resonance energy transfer.

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http://dx.doi.org/10.1016/j.niox.2016.09.002

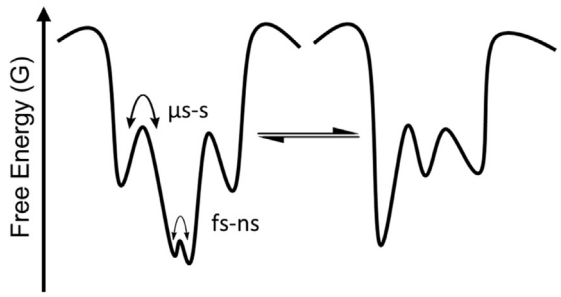

Fig. 1. Simplified two dimensional depiction of a multidimensional conformational landscape of a protein molecule. The 'valley' features of the landscape represent (quasi) stable conformational states that interconvert via higher energy barriers or 'hills' [10,11]. Temperature, pressure, mutagenesis, protein-protein interactions, reaction chemistry and ligand/inhibitor binding all influence the conformational landscape of a protein molecule (see text).

X-ray crystal structures of the individual NOS domains [18,26–30], along with recently published cryo-EM data [31–33], have helped to illustrate the structural organisation of this enzyme (Fig. 2). The three tissue-specific NOS isoenzymes are homodimeric proteins which bind and are activated by CaM. Each NOS reductase domain contains distinct FAD and FMN binding subdomains (Fig. 2A) and this domain is homologous to CPR, a microsomal membrane-bound diflavin oxidoreductase, which transfers electrons to a multitude of partner proteins, e.g. cognate cytochrome P450 enzymes (CYPs) [34,35]. Other members of the diflavin oxidoreductase family include methionine synthase reductase (MSR), a key enzyme in folate and methionine metabolism [36] and the reductase domain of the bacterial CYP cytochrome P450 BM3 (P450 BM3) [37].

The NOS reductase and oxygenase domains are connected by a CaM binding site. All three NOS isoforms bind to CaM, which is essential for NOS catalysis. However, the binding between both the

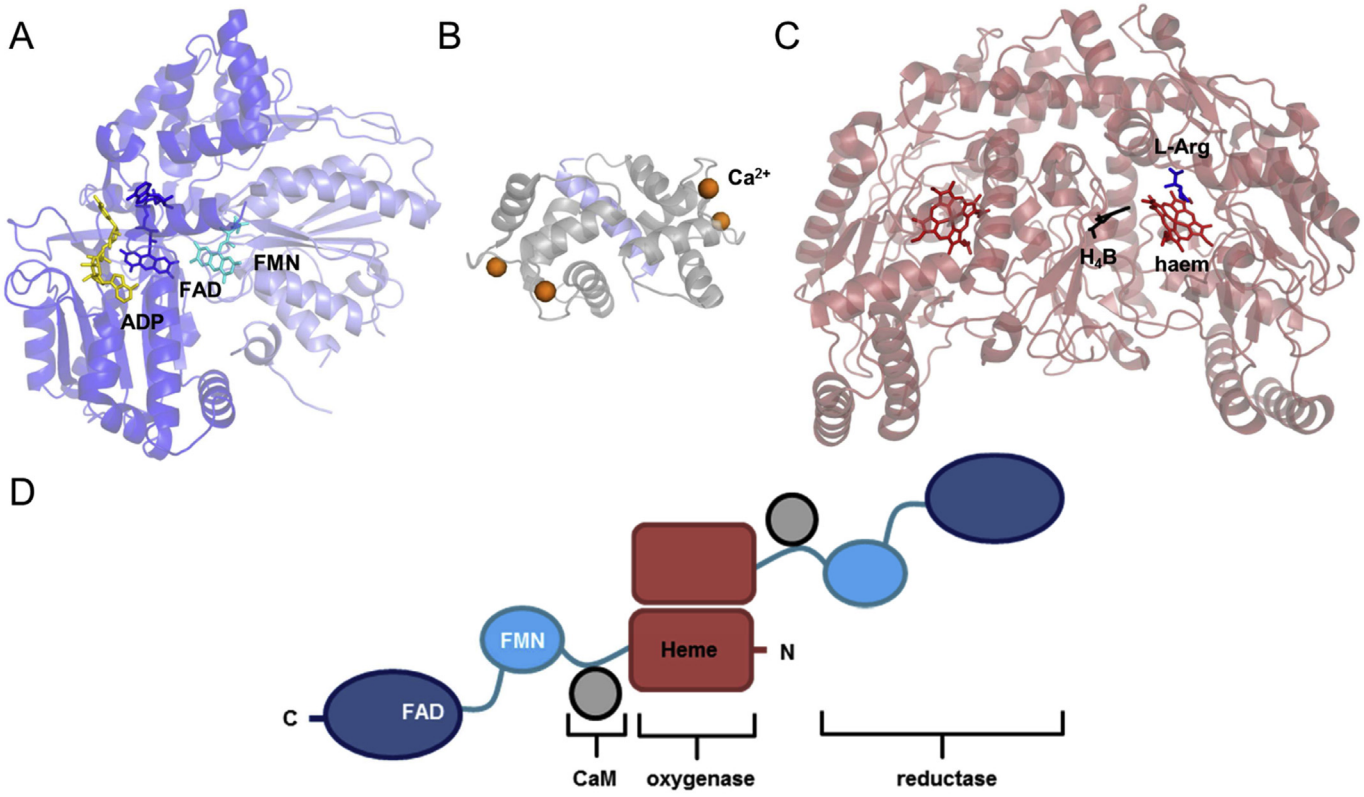

Fig. 2. Structure and molecular architecture of NOS. A) The structure of the $NADP^+$-bound neuronal NOS diflavin reductase domain (PDB ID 1TLL). B) The structure of holo-CaM bound to a nNOS-peptide (PDB ID 2O60). C) The crystal structure of the nNOS oxygenase domain dimer (PDB ID 1ZVL). CaM binds between the oxygenase and reductase domains of NOS. D) Structural organisation of the functional NOS dimer. The NOS FAD and FMN binding domains are shown in dark blue and light blue, respectively. CaM is shown in grey and the NOS oxygenase domain is shown in red.

biophysics of NOS [19–25]. Herein, as an alternative perspective, we present how recently published fluorescence spectroscopic data have helped in illustrating the nature of the NOS conformational landscape related to catalysis. We offer an overview of the information gathered from single molecule and ensemble fluorescence spectroscopy studies for the three tissue specific NOS isoenzymes and also for the related diflavin oxidoreductase cytochrome P450 reductase (CPR).

2. Structure of the NOS isoenzymes

Despite the lack of an atomistic structure of full length NOS, the constitutive NOS (cNOS) proteins [neuronal NOS (nNOS) and endothelial NOS (eNOS)] and CaM is Ca^{2+} dependent and reversible, while inducible NOS (iNOS)-CaM interactions occur regardless of intracellular calcium concentrations [20].

Many spectroscopic studies have shown the flux of electrons in NOS isoenzymes occurs from NADPH, through FAD and FMN cofactors, to the catalytic haem centre where NO is produced (see below). However, based on crystallographic data of the isolated NOS reductase domain in complex with $NADP^+$, where the FAD and FMN cofactors are in proximity, the electron transfer from reductase to oxygenase domains is thought not to be possible, due to the occluded location of the FMN [18]. This structure suggests that a