Journal of Inorganic Biochemistry xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

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

Insight into structural rearrangements and interdomain interactions related to electron transfer between flavin mononucleotide and heme in nitric oxide synthase: A molecular dynamics study

Yinghong Sheng [a,*], Linghao Zhong [b], Dahai Guo [c], Gavin Lau [a], Changjian Feng [d,*]

[a] Department of Chemistry & Physics, College of Arts & Sciences, Florida Gulf Coast University, 10501 FGCU Blvd. S., Fort Myers, FL 33965, USA
[b] Pennsylvania State University at Mont Alto, 1 Campus Drive, Mont Alto, PA 17237, USA
[c] Department of Bioengineering and Software Engineering, U.A. Whitaker College of Engineering, Florida Gulf Coast University, 10501 FGCU Blvd. S., Fort Myers, FL 33965, USA
[d] Department of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico, Albuquerque, NM 87131, USA

ARTICLE INFO

Article history:
Received 25 April 2015
Received in revised form 29 June 2015
Accepted 5 August 2015
Available online xxxx

Keywords:
Molecular dynamics
Nitric oxide synthase
Electron transfer
Heme
Calmodulin

ABSTRACT

Calmodulin (CaM) binding to nitric oxide synthase (NOS) enables a conformational change, in which the FMN domain shuttles between the FAD and heme domains to deliver electrons to the active site heme center. A clear understanding of this large conformational change is critical, since this step is the rate-limiting in NOS catalysis. Herein molecular dynamics simulations were conducted on a model of an oxygenase/FMN (oxyFMN) construct of human inducible NOS (iNOS). This is to investigate the structural rearrangements and the domain interactions related to the FMN–heme interdomain electron transfer (IET). We carried out simulations on the iNOS oxyFMN·CaM complex models in [Fe(III)][$FMNH^{-}$] and [Fe(II)][$FMNH^{\bullet}$] oxidation states, the pre- and post-IET states. The comparison of the dynamics and conformations of the iNOS construct at the two oxidation states has allowed us to identify key factors related to facilitating the FMN–heme IET process. The computational results demonstrated, for the first time, that the conformational change is redox-dependent. Predictions of the key interacting sites in optimal interdomain FMN/heme docking are well supported by experimental data in the literature. An intra-subunit pivot region is predicted to modulate the FMN domain motion and correlate with existence of a bottleneck in the conformational sampling that leads to the electron transfer-competent state. Interactions of the residues identified in this work are proposed to ensure that the FMN domain moves with appropriate degrees of freedom and docks to proper positions at the heme domain, resulting in efficient IET and nitric oxide production.

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1. Introduction

Nitric oxide (NO) is a key signaling molecule for vasodilation and neurotransmission at low concentrations and a defensive cytotoxin at higher concentrations [1,2]. Nitric oxide synthase (NOS) is the enzyme responsible for biosynthesis of NO from L-arginine. NO's availability is tightly regulated at the synthesis level by NOS. Deviant NO production by NOS is a major contributor to the pathology of often fatal diseases that currently lack effective treatments, including stroke [3]. To date, clinical NOS modulators still remain elusive. Before logically designing an effective therapeutic strategy by targeting NOS/NO, one must understand the mechanism of NOS regulation at the molecular level. Yet, there is still much unknown about the mechanism of tight regulation of NO production by NOS. It is thus of current interest to investigate regulation mechanisms of the NOS enzymes.

Mammalian NOS enzyme catalyzes the 5-electron oxidation of L-arginine (l-Arg) to NO and citrulline, utilizing NADPH and O_2 as co-substrates [1]. NOS is a redox enzyme consisting of multiple relatively rigid domains that are connected by flexible linkers. Each subunit of the NOS homo-dimer has two domains joined by a calmodulin (CaM) binding linker: a C-terminal electron-supplying reductase domain, which consists of smaller (sub)domains with binding sites for NADPH (the electron source), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN), and an N-terminal catalytic oxygenase domain with heme, tetrahydrobiopterin (H_4B), and arginine substrate binding sites forming the catalytic center for NO production (the terms 'oxygenase domain' and 'heme domain' are interchangeable). NOS's activity depends in equal measure on the reactions at the heme active site and on the overall dynamic structural rearrangements that enable the timely delivery of electrons to the active site (see below).

Three NOS isoforms, iNOS, eNOS and nNOS (inducible, endothelial, and neuronal NOS), achieve their biological functions by tight control of interdomain electron transfer (IET) process through interdomain interactions [4,5]. In particular, inter-subunit FMN–heme IET (Eq. (1)) is

* Corresponding authors.
E-mail addresses: ysheng@fgcu.edu (Y. Sheng), cfeng@unm.edu (C. Feng).

http://dx.doi.org/10.1016/j.jinorgbio.2015.08.006
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the essential step in coupling electron transfer in the reductase domain with NO synthesis in the heme domain by delivery of electrons required for O_2 activation at the catalytic heme site [6].

$$[\mathrm{Fe(III)}][\mathrm{FMN_{hq}}] \rightleftharpoons [\mathrm{Fe(II)}][\mathrm{FMNH^{\bullet}}] \quad (1)$$

The protonation state of the NOS flavin hydroquinone is not known yet, and FMN_{hq} is used to represent FMN hydroquinone in Eq. (1). The FMN–heme IET process is proposed to involve large scale motions of the FMN domain (Fig. 1) [5]. Starting from the NADPH–FAD/FMN state (i.e., electron-accepting input state), the FMN molecule receives an electron from the FAD center, and is reduced to FMN_{hq}. The FMN domain then moves away from the NADPH–FAD domain and migrates to the oxygenase domain (forming the output state) so that the FMN_{hq} can deliver an electron to the heme center, where Fe(III) is reduced to Fe(II) and the FMN center is converted to its semiquinone form $FMNH^{\bullet}$. Subsequently, the FMN domain moves away from the heme domain toward back to the NADPH–FAD domain (to shuttle another NADPH-derived electron). This FMN domain tethered shuttle model (Fig. 1) has been supported by recent kinetics [7–16], thermodynamic [17] and spectroscopic [18,19] results. Emerging evidence indicates that CaM activates NO synthesis in eNOS and nNOS by allowing the conformational change, and that CaM is also required for proper alignment of the FMN and heme domains [4,5]. However, the control mechanism underlying the large movement of the FMN domain between the NADPH–FAD and heme domains remain unclear. The precise role of CaM regulating this conformational change, especially how CaM interacts with the heme domains and constrains the motions of the FMN domain, is poorly understood [20].

A full-length NOS structure will help understand the NOS regulation mechanism. Unfortunately, the mobility of the NOS domains makes crystallization of the full-length enzyme very challenging, thus the structure of full-length NOS has not been obtained. Even if such crystal structures were available, the molecular mechanisms that promote function may still remain elusive, since NOS is a highly dynamic protein existing in a broad range of interconverting conformations [21,22]. The crystals can only stabilize some specific structural sub-states that define the functional state of the protein. Thus, X-ray crystallography alone will not solve the NOS structural problems and may only shed light on some of the static structural details without addressing the dynamics aspects.

Electron microscopy (EM) is a useful method for determining the structures of large proteins and protein complexes. In the last two years, four cryo-EM studies of the three full-length NOS isoforms were published [23–26], giving substantial insight into the molecular architecture of NOSs. This also attests an urgent need of elucidating conformational changes required for efficient electron transfer. However,

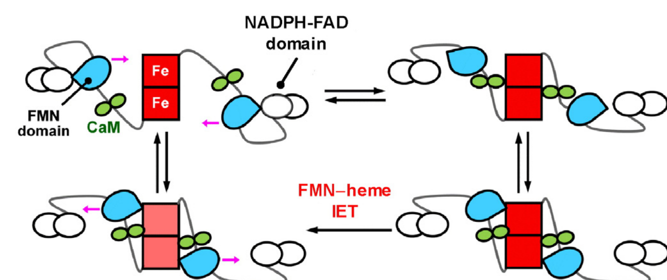

Fig. 1. The FMN domain tethered shuttle model (with the tethers corresponding to the interdomain FMN–heme and FAD–FMN connectors). The FMN domain (cyan) shuttles between the NADPH–FAD domain (white) and the heme-containing oxygenase domain (red). CaM (green) binding to eNOS/nNOS unlocks the NADPH–FAD/FMN domain interacting state (i.e., input state), thereby enabling the FMN domain to shuttle between the FAD and heme domains. The input state and FMN/heme domain interacting state (i.e., output state) are relatively well defined, while free FMN domain conformations also exist in between these two docked states.

structures determined by cryo-EM are of low resolution, and the classification of the conformational states in these works is categorized according to the perceived overall shapes of the entire dimer, but not the precise relative arrangement of the specific structural parts of the protein. At low resolutions (23 Å [25] and 60–74 Å [23]), cryo-EM cannot provide explicit information on the interactions between the FMN and heme domains. Moreover, the authors used negative-stain EM methods, which involved fixing the protein sample on a carbon-coated surface and treating it with a high-contrast heavy metal stain. While this is a powerful approach for observing protein complexes, in certain cases the grid surface can distort the sample and lead to a debatable asymmetry [23].

Magnetic resonance and fluorescence-based approaches represent a powerful complement to traditional structural biology methods and are now increasingly used to probe the NOS structure and function. The size of the NOS enzymes (~120–160 kDa per subunit) prevents application of NMR spectroscopy with methods available to date, although ^{13}C- and ^{15}N-labeled CaM proteins were recently used in the NMR studies of structure and dynamics of CaM bound to peptides corresponding to the CaM-binding linker in the NOS proteins [27,28]. Fluorescence resonance energy transfer approach can provide distance data, but there are known complexities when using the intrinsic fluorophores FMN and FAD since their fluorescence quantum yields depend on solvent accessibility and redox state. Tagging the protein domains with donor and acceptor fluorophores may be a better approach, but it needs to be tested if the bulky tags interfere with the NOS activity.

A clear understanding of the large NOS conformational change is critical, since this step is the rate-limiting in NO production [23]. While experimental approaches still face tremendous challenges at this moment, computational simulation can provide valuable information on the dynamics of enzyme structures, key residue–residue interaction evolution, and hence facilitate the understanding of the roles of inter-domain interactions and protein dynamics in NOS regulation. Up to date, no computational simulation has been reported to elucidate the domain motions in NOS. The time scale for the interconverting between input and output states has not been experimentally measured, but should be at milliseconds to seconds range since the rates of similar FMN domain binding of and release from the FAD domain are estimated to be around 10 s^{-1} [29,30]. Such long time scale is beyond current computational simulation capacity.

We thus focused on studying the dynamics of the NOS output state using models of a CaM-bound bi-domain oxygenase/FMN (oxyFMN) construct. This construct is a minimal electron transfer complex designed to favor the interactions between the FMN and heme domains [31], and biochemical and kinetics studies demonstrated that it is a valid representation of the NOS output state for NO production [4,5]. In the present work, we have conducted molecular dynamics (MD) simulations on the two redox states before and after the IET process (Eq. (1)). Based on the initial docking model for a human iNOS oxyFMN construct, MD simulations were carried out to elucidate the structural rearrangements and the interactions between domains. Specifically, we examined role of redox states change (due to the IET) in the conformational changes. The comparison of the dynamics and structures of the NOS oxyFMN construct at the two oxidation states has allowed us to identify key factors related to facilitating the FMN–heme IET process. The predictions of key interacting sites are well supported by experimental data in the literature, and new sites have also been identified from the simulation. These computational results have provided new insight into the dynamic interactions in regulating the NOS electron transfer and function.

2. Methods

2.1. Initial structure construction

The initial docked structures of human iNOS oxyFMN·CaM construct were built using the crystal structures of human iNOS heme domain

Please cite this article as: Y. Sheng, et al., Insight into structural rearrangements and interdomain interactions related to electron transfer between flavin mononucleotide and..., J. Inorg. Biochem. (2015), http://dx.doi.org/10.1016/j.jinorgbio.2015.08.006

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