FEBS Letters 587 (2013) 297–301

journal homepage: www.FEBSLetters.org

Calmodulin-induced structural changes in endothelial nitric oxide synthase

Anthony Persechini *, Quang-Kim Tran, D.J. Black, Edward P. Gogol

Division of Molecular Biology and Biochemistry and Division of Cell Biology and Biophysics, University of Missouri at Kansas City, 5007 Rockhill Rd, Kansas City, MO 64110-2499, United States

ARTICLE INFO

Article history:
Received 20 November 2012
Revised 10 December 2012
Accepted 11 December 2012
Available online 22 December 2012

Edited by Peter Brzezinski

Keywords:
Nitric oxide synthase
Calmodulin
Enzyme regulation
Enzyme structure

ABSTRACT

We have derived structures of intact calmodulin (CaM)-free and CaM-bound endothelial nitric oxide synthase (eNOS) by reconstruction from cryo-electron micrographs. The CaM-free reconstruction is well fitted by the oxygenase domain dimer, but the reductase domains are not visible, suggesting they are mobile and thus delocalized. Additional protein is visible in the CaM-bound reconstruction, concentrated in volumes near two basic patches on each oxygenase domain. One of these corresponds with a presumptive docking site for the reductase domain FMN-binding module. The other is proposed to correspond with a docking site for CaM. A model is suggested in which CaM binding and docking position the reductase domains near the oxygenase domains and promote docking of the FMN-binding modules required for electron transfer.

1. Introduction

The nitric oxide synthases (NOS) catalyze formation of NO and L-citrulline from L-arginine and oxygen, with NADPH as the electron donor [1]. The three major mammalian forms of the enzyme are commonly referred to as iNOS (inducible), nNOS (neuronal) and eNOS (endothelial) [2,3]. All of these are functional homodimers of 130–160 kDa monomers. Each monomer contains a reductase and oxygenase domain joined by a ~35 amino acid linker sequence containing a calmodulin (CaM)-binding domain [1]. The interface between the two oxygenase domains appears to be responsible for maintenance of the enzyme dimer in solution [4–7]. Each reductase domain contains an FMN-binding and FAD–NADPH-binding module, joined by a ~20 amino acid linker [8]. Electron transfer during catalysis occurs in *trans*, with electrons flowing from the reductase domain of one monomer to the heme reaction center in the oxygenase domain of the other [9–11]. This appears to involve movement of the FMN modules between their respective NADPH–FAD modules and docking sites on the oxygenase domains [12,13].

All three enzyme isoforms have negligible synthase activity in the absence of CaM, which is bound with significant affinity to eNOS and nNOS only in its Ca^{2+}-bound form, and to iNOS in both its Ca^{2+}-free and Ca^{2+}-bound forms [14–17]. Various observations suggest that CaM activates synthase activity both by increasing the efficiency of electron transfer within the reductase domains, from NADPH to FMN via FAD, and by increasing the efficiency of electron transfer to the heme reaction centers via reduced FMN, in part by mobilizing the FMN modules [12,13,18–21]. Although crystal structures have been determined for the dimeric eNOS oxygenase domain [7], and for a dimeric form of the nNOS reductase domain [8], no structures have been published thus far for an intact NOS isoform.

In this paper we present solution structures of full-length CaM-bound and CaM-free bovine eNOS at a nominal resolution of ~25 Å, derived by reconstruction from cryo-electron micrographs of the enzyme in vitreous ice. These structures suggest significant new insights to the structural relationship between the reductase and oxygenase domains, and how this relationship is affected by CaM to produce synthase activation.

2. Materials and methods

A mutant of bovine eNOS containing a phosphomimetic S1179D substitution was used for these investigations because its maximal CaM-dependent synthase activity is twice that of the native protein, suggesting that in the presence of CaM more is in the fully active conformation [22]. This protein was expressed in *Escherichia coli* and purified as described elsewhere [22,23]. The vertebrate CaM amino acid sequence, encoded by a rat cDNA, was expressed

Abbreviations: NOS, nitric oxide synthase; eNOS, endothelial NOS; iNOS, nNOS, inducible and neuronal NOS; CaM, calmodulin

* Corresponding author.
E-mail address: persechinia@umkc.edu (A. Persechini).

0014-5793/$36.00
http://dx.doi.org/10.1016/j.febslet.2012.12.012

in *E. coli* and purified as described previously [24]. Immediately prior to preparation of samples for microscopy, 50 μl aliquots of purified eNOS were thawed and analyzed by size exclusion chromatography on a Superdex 200 HR 10/30 column at 4 °C in a buffer containing 25 mM Tris–HCl, pH 7.4, 100 mM KCl, 1 mM $CaCl_2$ and 1 mM dithiothreitol. Peak fractions previously shown to correspond with the intact dimeric enzyme were pooled, and the monomer concentration of eNOS was determined based on optical absorbance at 397 nm [23]. Prior to freezing on grids, the enzyme was diluted to a concentration of 30–150 nM in column buffer, with or without a 1.5-fold molar excess of $(Ca^{2+})_4$–CaM. The apparent K_D for the $(Ca^{2+})_4$–CaM–eNOS complex is below 1 nM [25], so under these conditions the enzyme should be saturated with CaM.

Fenestrated carbon films (Quantifoil Micro Tools GmbH) subjected to glow-discharge were used for application, blotting and freezing of proteins in liquid ethane. The samples were stored in liquid nitrogen until loading into a Gatan 626 holder and imaging with a JEOL 1200 IIX electron microscope at 100 kV, using minimal dose protocols. Micrographs were recorded on Kodak SO163 film, using defocus values between 1.2 and 3 μm, and digitized using a Hi-Scan drum scanner with a 5 Å pixel on the specimen. Individual particles were selected from images wavelet-filtered to increase contrast, and the coordinates thus obtained were used to extract unfiltered particle images in 40 × 40 pixel (200 × 200 Å) boxes. The CaM-free and CaM-bound eNOS data sets each contain ~25,000 images. Phase correction of the particle images was based on defocus values estimated using the ACE software package [26].

Euler angles were assigned to each image based on projection-matching to de novo common-lines initial models. These were generated from reference-free image averages of both data sets sorted into classes by iterative multivariate statistical analysis with the EMAN software package [27]. An initial model derived in this manner for each data set (±CaM) was used to initiate iterative projection-matching in 7° angular increments with twofold symmetry imposed, using the EMAN software. A cutoff for correlation with model projections eliminated approximately 35% of the particles from the data. Convergence was reached within five to eight rounds of refinement based on round-to-round resolution calculations. To test model dependence, the two initial models (±CaM) were exchanged, and the final reconstructions of each dataset were visually indistinguishable from those initiated with the "correct" model. The amplitudes of the reconstructions were corrected in defocus groups guided by a solution scattering curve of a similar-sized protein dimer, fatty acid synthase [28], at resolutions between 100 and 25 Å. The resolutions of the reconstructions were calculated by comparison of Fourier shell coefficients (Fig. 1A), both yielding a limit of ~25 Å at a 0.5 correlation value. Characteristic projections of both reconstructions compare well with reference-free class averages of the phase-corrected data derived using the refine2d component of the EMAN software package (Fig. 1B and C).

Fitting and correlation of a simulated 25 Å resolution density map derived from the oxygenase domain dimer crystal structure (PDB ID = 1FOP) [7] was performed using the Chimera molecular graphics package [29]. The DelPhi software suite [30] was used to calculate the electrostatic potential surface for the crystal structure displayed in Fig. 1C. A homology model for the eNOS FMN module was derived using standard methods from the nNOS reductase domain dimer crystal structure (PDB ID = 1TLL) [8].

3. Results

The final CaM-free and CaM-bound eNOS reconstructions are displayed in Fig. 2A and B as volumes enclosed at the level of steepest density drop-off, which corresponds with the apparent surface of the protein. A simulated 25 Å density map (colored red) for the oxygenase domain dimer has been fitted to the CaM-free and CaM-bound reconstructions [7]. The reconstructions and simulated oxygenase density are also represented in the figure as cross-sectional contour plots taken at the levels indicated in the reconstructions by lines 1–4. The red lines in the contour plots enclose the simulated density for the fitted oxygenase domain dimer.

Both the CaM-free and CaM-bound reconstructions accommodate the oxygenase dimer crystal structure, but neither can accommodate the reductase domains, which correspond to ~60% of the 260 kDa mass of the eNOS dimer. An excellent correspondence can be obtained between the CaM-free reconstruction and the simulated oxygenase domain density (correlation = 0.93). However, the reductase domains appear to have been lost by the image averaging inherent in the reconstruction process, indicating that they are highly mobile with respect to the oxygenase domains. The small amount of volume near the the "top" of the CaM-free reconstruction that is unaccounted for by the simulated oxygenase density may correspond with ~65 amino acids missing from the N-termini of the crystal structure [7].

In the case of the CaM-bound eNOS, the highest-density portions of the reconstruction also appear to match the simulated oxygenase density, but extensive peripheral density cannot be accounted for by

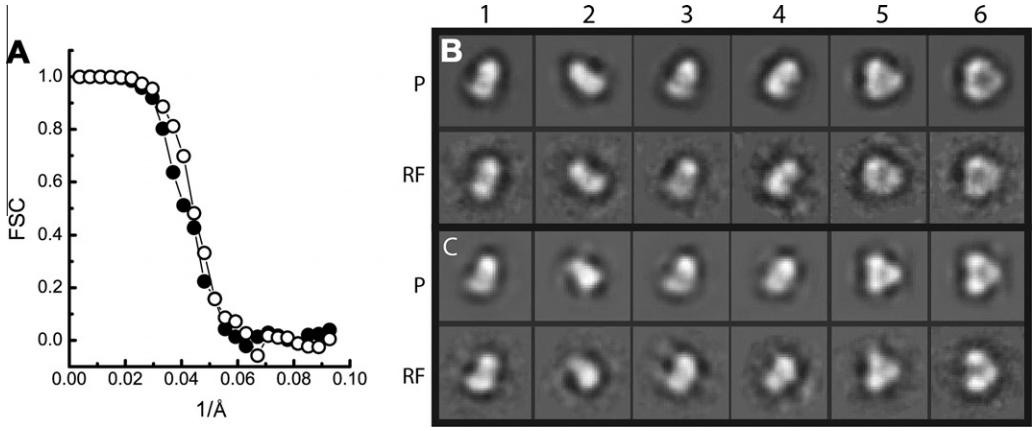

Fig. 1. Analysis of reconstructions. (A) Fourier shell correlation (FSC) plots for the CaM-free (○) and CaM-bound (●) reconstructions derived from odd and even images in the projection classes. Nominal resolutions correspond to the reciprocal of the shell where the FSC value equals 0.5, which yields a limiting value of ~25 Å for both reconstructions. (B & C) Characteristic projections (P) of the CaM-free (B) and CaM-bound (C) eNOS reconstructions compared with reference-free class averages (RF) derived directly from the data. Projections of the reconstructions before amplitude correction were generated after aligning them (corr = 0.96) using the Chimera software [29]. Reference-free class averages were derived from phase-corrected images as described in Section 2.