Biochimica et Biophysica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Characterization of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*: Enzymatic activity and active site structure ☆

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ARTICLE INFO

Article history:
Received 21 November 2013
Received in revised form 10 February 2014
Accepted 15 February 2014
Available online xxxx

Keywords:
Nitric oxide reductase
Heme
Non-heme metal
Cytochrome *c* oxidase
Resonance Raman
Proton transfer

ABSTRACT

Nitric oxide reductase (NOR) catalyzes the reduction of nitric oxide to generate nitrous oxide. We recently reported on the crystal structure of a quinol-dependent NOR (qNOR) from *Geobacillus stearothermophilus* [Y. Matsumoto, T. Tosha, A.V. Pisliakov, T. Hino, H. Sugimoto, S. Nagano, Y. Sugita and Y. Shiro, *Nat. Struct. Mol. Biol.* 19 (2012) 238–246], and suggested that a water channel from the cytoplasm, which is not observed in cytochrome *c*-dependent NOR (cNOR), functions as a pathway transferring catalytic protons. Here, we further investigated the functional and structural properties of qNOR, and compared the findings with those for cNOR. The pH optimum for the enzymatic reaction of qNOR was in the alkaline range, whereas *Pseudomonas aeruginosa* cNOR showed a higher activity at an acidic pH. The considerably slower reduction rate, and a correlation of the pH dependence for enzymatic activity and the reduction rate suggest that the reduction process is the rate-determining step for the NO reduction by qNOR, while the reduction rate for cNOR was very fast and therefore is unlikely to be the rate-determining step. A close examination of the heme/non-heme iron binuclear center by resonance Raman spectroscopy indicated that qNOR has a more polar environment at the binuclear center compared with cNOR. It is plausible that a water channel enhances the accessibility of the active site to solvent water, creating a more polar environment in qNOR. This structural feature could control certain properties of the active site, such as redox potential, which could explain the different catalytic properties of the two NORs. This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

1. Introduction

Microbial denitrification, in which nitrate (NO_3^-) is stepwise reduced to dinitrogen (N_2) through nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O), is an example of anaerobic respiration. Some soil bacteria can produce bio-energy, such as ATP, by denitrification. Nitric oxide reductase (NOR) is a membrane-integrated metalloprotein, which, as a part of the denitrification process, catalyzes the reduction of NO to N_2O by the use of two protons and two electrons ($2NO + 2H^+ + 2e^- \rightarrow N_2O + H_2O$). This reaction involves the important chemical process of N–N bond formation in the global nitrogen cycle, thus enabling the eventual release of N_2 into the atmosphere. NOR is also an attractive target of study in Earth science, as N_2O, the product of the NOR-catalyzed reaction, is both an ozone (O_3) depleting substance and a greenhouse gas with 310 times potency of carbon dioxide (CO_2) [1].

On the basis of similarities in the amino acid sequence, NOR has been thought to share the same ancestor protein with aerobic terminal oxidases such as cytochrome *c* oxidase (CcO) [2–4]. CcO catalyzes the reduction of O_2 to water by four protons and four electrons ($O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$) at a heme iron and copper (Cu_B) binuclear center, and, coupled with the catalytic reaction, pumps protons across the membrane. The generated electrochemical proton gradient is then used for ATP synthesis. High resolution crystal structures, along with extensive biochemical and biophysical studies of CcOs provide a detailed picture of the mechanism involved in the proton pumping in a process coupled with the O_2 reduction reaction [5–7]. Since NOR exhibits NO reduction activity without proton pumping, a knowledge of the structural characteristics of NOR is crucial for elucidating functional conversion – for example, substrate selectivity and proton pumping ability – of these respiratory enzymes during molecular evolution.

Abbreviations: NOR, bacterial nitric oxide reductase; qNOR, quinol-dependent NOR; cNOR, cytochrome *c*-dependent NOR; CcO, cytochrome *c* oxidase; DDM, *n*-dodecyl-β-D-maltoside; OG, *n*-octyl-β-D-glucoside; PMS, phenazine methosulfate.

☆ This article is part of a Special Issue entitled: 18th European Bioenergetic Conference.

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http://dx.doi.org/10.1016/j.bbabio.2014.02.017
0005-2728/

Three classes of NOR have been identified in bacteria and archaea. The first class, called cNOR, is a two subunit enzyme that is observed only in denitrifying bacteria, and uses soluble proteinous electron donors, such as cytochrome *c*, for the catalytic reaction [8]. The second class, known as quinol-dependent NOR (qNOR), is a single-subunit enzyme that is observed in non-denitrifying pathogenic bacteria as well as in denitrifying bacteria and archaea [8–10]. The physiological role of qNOR of pathogenic bacteria is to detoxify NO produced by the immune system of the host [11,12]. qCu_ANOR which was isolated from denitrifying *Bacillus azotoformans* and has di-copper site at the hydrophilic domain represents the third class [13], although little is known regarding this enzyme, with not even sequence information available. Of these NOR enzymes, cNOR is the most extensively studied [14–17]. We published the first crystal structure of NOR, cNOR from *Pseudomonas aeruginosa* in 2010 [18]. The NO reduction site consists of a heme (designated as heme b_3) iron and a non-heme iron (Fe_B), and is structurally related to the heme/Cu_B binuclear center in CcO. Fe_B has a distorted trigonal–bipyramidal geometry with three histidine ligands and one glutamate ligand. From the *P. aeruginosa* cNOR structure and structure-based molecular dynamics simulation, no evidence for the existence of a proton pumping pathway was found, but potential pathways for transferring catalytic protons from the periplasmic side were identified [18,19]. Overall, this is consistent with the non-electrogenicity of the NO reduction reaction catalyzed by cNOR [17,20].

We have also recently reported the crystal structure of *Geobacillus stearothermophilus* qNOR [21]. Compared with *P. aeruginosa* cNOR, the overall structure of *G. stearothermophilus* qNOR is similar, but no obvious proton transfer pathway from the extracellular side was found. Instead, a water-filled hydrophilic channel from the cytoplasmic side to the binuclear center was observed. Structure-based mutagenesis and molecular dynamics simulation studies suggested that the water channel could possibly function as a proton transfer pathway for the catalytic reaction. This finding for *G. stearothermophilus* qNOR is very surprising, as, in two NOR enzymes performing the same enzymatic reaction, catalytic protons seem to be supplied to the active site from differing directions.

However, no supporting data is available for this novel finding. Only a few structural and functional studies on qNORs have been reported so far [22–24], and, as a result, the functional properties of qNOR are poorly understood as compared with the well-studied cNOR. In addition (and unfortunately), our *G. stearothermophilus* qNOR expressed in *Escherichia coli* and purified using *n*-dodecyl-β-D-maltoside (DDM) as a detergent was isolated as a mixture of active and inactive forms in a ratio of about 3:7. Atomic absorption analysis showed that in the active enzyme, a non-heme iron, Fe_B, is present at the binuclear active center, as in the case of *P. aeruginosa* cNOR, whereas, in the inactive form, the Fe_B had accidentally been replaced by Zn (Zn_B) [21]. In an attempt to improve the final yield of the active enzyme, more supplementary iron was used in expression cultures, and alternative detergents applied in the purification procedure; however, the purified sample still resulted in a similar population (~70%) of the inactive Zn_B-bound qNOR. When using *n*-octyl-β-D-glucoside (OG) as a detergent (for crystallization purposes), the active Fe_B-bound form was precipitated, leaving only the inactive Zn_B form to be crystallized [21]. Our crystal structure of *G. stearothermophilus* qNOR was thus that of an inactive Zn_B form. Therefore, we now report on the characterization of the active Fe_B form of *G. stearothermophilus* qNOR in this study. We examined the catalytic properties, in particular the proton uptake mechanism, of *G. stearothermophilus* qNOR by measuring the enzymatic activity under different pH values, because the proton transfer pathway would be expected to be different between *G. stearothermophilus* qNOR and cNOR on the basis of their structural comparison [18,19,21]. In addition to functional properties, the structure of the heme environment of *G. stearothermophilus* qNOR was characterized by resonance Raman spectroscopy, in an attempt to elucidate structure–function relationships. A comparison of findings for *G. stearothermophilus* qNOR with those for *P. aeruginosa* cNOR permits the structural and functional differences in these two classes of NOR to be determined and discussed.

2. Materials and methods

2.1. Sample preparation

The expression and purification of *G. stearothermophilus* qNOR followed a previous method [21]. Briefly, pET-22 plasmid encoding qNOR with a C-terminal His-tag was transformed into an *E. coli* Rosetta 2 DE3 (Novagen) strain. The *E. coli* was grown in 2xTY media with $FeSO_4$ and δ-aminolevulinic acid, with expression of qNOR induced by IPTG. The harvested cells were lysed, and the membrane fraction was isolated. After solubilization in 2% Triton X-100, the solubilized fraction was loaded onto a Ni-NTA column. The column was washed with buffer containing 0.05% DDM (Dojindo) or 1% OG (Dojindo) to exchange the detergent. The qNOR fraction was eluted with 300 mM imidazole, and then further purified using a Superdex 200 column. Fractions with $A_{Soret}/A_{280} > 0.7$ were employed in further experiments. The concentration of the purified sample was determined using a molar extinction coefficient of 206 mM^{-1} cm^{-1} at the Soret peak. Analysis of metal content by inductively coupled plasma atomic emission spectroscopy showed a complete occupation of zinc at the non-heme metal site with OG as the detergent, whereas the non-heme metal site was occupied by either iron (~30%) or zinc (~70%) when DDM was used [21]. Because the sample with zinc at the non-heme metal site was enzymatically inactive, qNOR prepared with OG was used only for spectroscopic characterization of the active site, [21]. The purified sample was stored at −80 °C until use.

The cNOR used in this study was purified from anaerobically cultured *P. aeruginosa*. Preparation of membranes and purification of cNOR were performed as previously described [18]. The purified fractions with $A_{Soret}/A_{280} > 1.5$ were used in further experiments. The concentration of the purified sample was determined using the molar extinction coefficient of 300 mM^{-1} cm^{-1} at the Soret peak [18]. The sample was stored at −80 °C until use.

2.2. NO reduction activity measurements

NO reduction activity was measured using a Clark-type electrode equipped with an ISO-NO mark II system (WPI). The anaerobic assay mixture (0.9 mL) consisted of 50 mM Tris buffer (pH 7.0), 0.05% DDM, 100 mM D-glucose, 10 μg/mL glucose oxidase, and 10 μg/mL catalase. For the pH dependence measurements, Bis-Tris, Tris and CAPS buffers were used for pH 6.0, 7.0–9.0, and 10.0, respectively. As an electron transfer system, 1 mM sodium ascorbate and 100 μM phenazine methosulfate (PMS) were used. NO saturated buffer was added to a final concentration of 10–40 μM. Once a stable electrode response was observed, the addition of NOR (final concentration: 0.2–0.5 μM) initiated the NO reduction reaction. NO reduction rates were calculated from the difference in the slope of the activity trace before and after the addition of the enzyme. Although substrate inhibition was reported in cNOR from *Paracoccus denitrificans* [25], substrate inhibition was not observed under the current assay condition. For qNOR, the activity was estimated relative to the Fe_B-containing active enzyme (30% of total enzyme). The measurements were carried out at 40 °C (for qNOR) or 20 °C (for cNOR). The NO reduction rates were measured at different pH values, ranging from pH 6.0 to 10.0 (the ascorbate/PMS electron transfer system does not function effectively at a pH of less than 5.0), and determined from at least three independent measurements.

2.3. Stopped flow measurements

The reduction of the qNOR or cNOR by the ascorbate/PMS electron transfer system was monitored by a rapid-scanning system equipped with a stopped flow apparatus (Unisoku, RSP-1000) at

Please cite this article as: E. Terasaka, et al., Characterization of quinol-dependent nitric oxide reductase from *Geobacillus stearothermophilus*: Enzymatic activity and active site structure, Biochim. Biophys. Acta (2014), http://dx.doi.org/10.1016/j.bbabio.2014.02.017