Journal of Electroanalytical Chemistry 693 (2013) 105–113

Contents lists available at SciVerse ScienceDirect

Journal of Electroanalytical Chemistry

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

Induced peroxidase activity of haem containing nitrate reductases revealed by protein film electrochemistry

Catarina Coelho [a], Jacopo Marangon [a], David Rodrigues [a], José J.G. Moura [a], Maria João Romão [a], Patrícia M. Paes de Sousa [a,*], Margarida M. Correia dos Santos [b,*]

[a] REQUIMTE/CQFB, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[b] Centro de Química Estrutural, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal

ARTICLE INFO

Article history:
Received 2 September 2012
Received in revised form 17 January 2013
Accepted 24 January 2013
Available online 9 February 2013

Keywords:
Periplasmic nitrate reductase
Respiratory nitrate reductase
Direct voltammetry
Peroxidase activity

ABSTRACT

Direct voltammetry of adsorbed redox enzymes at pyrolytic graphite electrodes has shown to be very useful to probe the catalytic activity of several nitrate reductases in the presence of nitrate. In this work we demonstrated that in some cases an electrode-induced haem alteration leads to a loss of nitrate reductase activity.

Nitrate reductases are key enzymes in the biological nitrogen cycle. In particular, NapAB from *Cupriavidus necator* has an important role in the scavenging of nitrate and NarGHI from *Marinobacter hydrocarbonoclasticus* 617 is essential for the anaerobic respiration. These enzymes present haem groups among their redox centres, which are essential for the electron transfer from the reducing to the oxidising substrate. When adsorbed at graphite electrodes, both enzymes displayed a non-turnover signal corresponding to a one-electron redox process, with formal reduction potentials at pH 7.6 of −159 mV and −139 mV vs. SHE for Nap and Nar, respectively. Both enzymes displayed peroxidase activity at a potential close to that of the non-turnover response. Experiments with the whole enzymes and the haem free NapA from *Desulfovibrio desulfuricans* and NarGH from *M. hydrocarbonoclasticus* 617 were a valuable tool to get information about the cofactor undergoing electron transfer. We confirmed that this behaviour is related with the haems present in subunit B and subunit I of NapAB and NarGHI, respectively.

1. Introduction

Nitrate reductases (NRs) are key enzymes in the biological nitrogen cycle. They are responsible for reducing nitrate to nitrite in a two-electron (two-proton) redox process, with release of one water molecule, according to the reaction:

$$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O \qquad E^0 = +420\,mV\ vs.\ SHE$$

Prokaryotic nitrate reductases constitute a broad group of enzymes, belonging to the dimethyl sulfoxide (DMSO) reductase family of molybdenum-containing enzymes [1,2]. NRs can be classified according to their localization in the cell, function and molecular properties of the active site as periplasmic (Nap), respiratory (Nar) and assimilatory (Nas) nitrate reductases (Fig. 1).

In the case of Nap several functions have been proposed, such as dissipation of excess cellular reductant during growth on reduced carbon substrates and scavenging of nitrate [3]. Several crystal structures of periplasmic nitrate reductases have been reported in the past decade: the monomeric NapA from the sulphate reducing bacteria *Desulfovibrio desulfuricans* (*Dd*) ATCC 27774 [4]; the individual NapA subunit from *Escherichia coli* (*Ec*) [5]; and the heterodimeric NapAB complexes from *Rhodobacter sphaeroides* (*Rs*) [6] and *Cupriavidus necator* (*Cn*) [7]. With the exception of *Dd* NapA, the majority of periplasmic nitrate reductases consist of two different subunits (NapA and NapB), tightly bound in the case of *Cn* NapAB. The large catalytic subunit (NapA) contains the molybdenum active site cofactor, a molybdopterin guanine dinucleotide (MoCo), plus one [4Fe–4S] cluster. The MoCo is accessible through a funnel-like cavity coated with a few charged residues that favour the binding and orientation of charged substrate molecules. The small subunit (NapB) contains two *c*-type haems involved in electron transfer. The NapB haems are almost parallel to each other, with a Fe–Fe distance of approximately 10 Å. The nearest iron atom from the [4Fe–4S] center is 14 Å away from haem I.

The respiratory nitrate reductases (NarGHI) are membrane bound proteins with a central role in the anaerobic respiration, where the nitrate reduction is coupled with the generation of the proton motive force by protons translocation to the periplasm [8]. All Nars isolated so far are heterotrimeric enzymes constituted

* Corresponding authors. Tel.: +351 212948300x10957; fax: +351 212948550 (P.M. Paes de Sousa), tel.: +351 218419272; fax: +351 218464455 (M.M. Correia dos Santos).

E-mail addresses: patricia.sousa@fct.unl.pt (P.M. Paes de Sousa), mcsantos@ist.utl.pt (M.M. Correia dos Santos).

1572-6657/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jelechem.2013.01.030

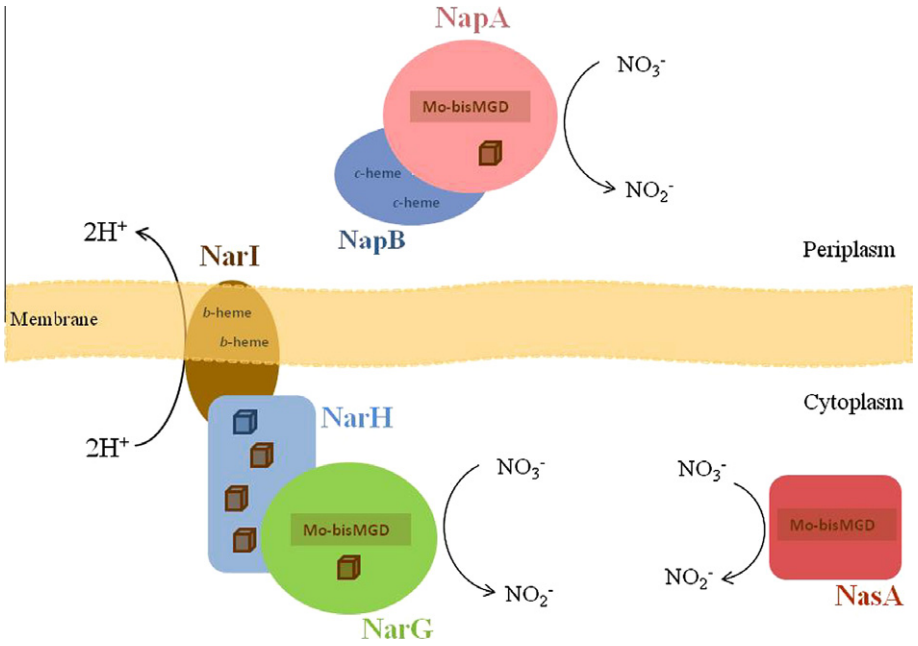

Fig. 1. Schematic representation of the localization of nitrate reductases in prokaryotic cells: NarGHI anchored to the membrane, NapAB in the periplasm, and Nas in the cytoplasm. The brown cubes represent the [4Fe–4S] centres and the blue cube the NarH [3Fe–4S] cluster. Nas is very diverse in terms of number and type of electron transfer centres for different organisms, and only NasA is represented. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by the subunits NarG (112–140 KDa), NarH (52–64 KDa) and NarI (19–25 KDa). The subunits NarG and NarH are located in the cytoplasm and are anchored to the membrane through the subunit NarI. The crystal structures available are for the oxidized form of the *Ec* NarGH [9] and *Ec* NarGHI [10]. The main subunit NarG hosts the MoCo and a [4Fe–4S] cluster designated as FeS0, and the NarH subunit contains one [3Fe–4S] (FeS4) and three [4Fe–4S] clusters (FeS1-3). The membrane subunit NarI contains two *b*-type haems termed b_P (proximal) and b_D (distal), which are involved in the quinolinic pool (MQH_2) oxidation and proton translocation across the membrane. A distance of 12–14 Å divides each redox cofactor allowing the electron transfer through the system.

Unlike spectroscopic techniques, dynamic electrochemical methods are not able to provide structural information. Yet, the use of voltammetric techniques is well proved nowadays in unravelling important aspects of the chemistry of metalloenzymes, allowing *in situ* measurements of the reduction potentials, together with the acquisition of information about relevant parameters of coupled reactions, including catalysis [11]. Most studies of direct electrochemistry of these molecules use the concept of protein film voltammetry (PFV) [12]. PFV involves a biocompatible adsorption of the protein onto the electrode surface, in such a way that a direct electrochemical communication between the enzyme and the electrode is established.

To date, several membrane-bounded (Nar) and periplasmic nitrate (Nap) reductases have been studied by direct electrochemistry, with the protein adsorbed onto an electrode: NarGH from *Paracoccus pantotrophus* (*Pp*) [13], NarGH from *Marinobacter hydrocarbonoclasticus* 617 (*Mh*) [14] and NarGHI *from E. coli* [15]; NapAB from *Pp* [16] and NapAB from *R. sphaeroides* [17]. The cytoplasmic assimilatory nitrate reductase NarB from *Synechococcus* sp. PCC 7942 was also studied using the same approach [18]. It was shown that these enzymes share a similar behaviour: non-turnover signals are never observed but in the presence of nitrate a cathodic catalytic current is always developed indicative of the electrocatalytic reduction of nitrate by the enzyme. As the electrode potential is taken more negative, catalytic activity for nitrate goes through a maximum at a given potential and then decreases and eventually levels off or, as is the case of *Mh* NarGH, a boost in activity (second sigmoidal component) is verified. The reason for these behaviours is still unclear and how unambiguous this is observed on the voltammetric waves may depend on nitrate concentration. Maximums are more clearly seen for low nitrate concentrations and they exist both in the forward and the reverse potential sweeps. For the homologous NapAB proteins from *P. pantotrophus* and *R. sphaeroides*, depending on the experimental conditions (pH and T) maximum catalytic activity occurs in the range of −100 to −200 mV [16,17,19–21]. It is difficult or not possible to relate the potential at the onset of catalysis with the redox potentials of the Mo cofactor. In the case of *Pp* NapAB these values are not known. For *Rs* NapAB the reduction potentials of the Mo cover a large potential range, −225 mV for Mo(IV)/Mo(V) and +550 mV for Mo(V)/Mo(VI) [6,22]. However, the observance of catalytic currents in the presence of nitrate indicates that electrons are flowing between the substrate and the electrode via the active site and so the electrocatalytic behaviour observed must be related with the redox chemistry of the MoCo. A similar behaviour was observed for *Ec* NarGHI, with the maximum of catalytic activity occurring at −25 mV while the reduction potentials of the couples Mo(V)/Mo(VI) and Mo(IV)/Mo(V) are +200 mV and +100 mV [15,23].

In this work we report a voltammetric study of two nitrate reductases, NapAB from *C. necator* and NarGHI from *M. hydrocarbonoclasticus* 617, formerly known as *Pseudomonas nautica* 617. The redox potentials of some of the metal cofactors of *Cn* NapAB were determined by spectrophotometric redox titrations, namely +50 mV for haem II (the more exposed haem), +160 mV for haem I and −15 mV for the [4Fe–4S] cluster [7]. The respiratory nitrate reductase (NarGHI) receives electrons from the oxidation of MQH_2 or UQH_2 [24]. The electrons are then transferred through the redox cofactors (b_D, b_P, FeS4, FeS3, FeS2, FeS1, FeS0, Mo-bis-MGD). The redox potentials of the cofactors are not known for *Mh* NarGHI, but they have been determined by UV–Vis- and EPR-mediated potentiometry in the case of the *E. coli* enzyme [23,25,26], which presents a high homology with *Mh* 617. The