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The solution structure of the soluble form of the lipid-modified azurin from *Neisseria gonorrhoeae*, the electron donor of cytochrome *c* peroxidase

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ABSTRACT

Neisseria gonorrhoeae colonizes the genitourinary track, and in these environments, especially in the female host, the bacteria are subjected to low levels of oxygen, and reactive oxygen and nitrosyl species. Here, the biochemical characterization of *N. gonorrhoeae* Laz is presented, as well as, the solution structure of its soluble domain determined by NMR. *N. gonorrhoeae* Laz is a type 1 copper protein of the azurin-family based on its spectroscopic properties and structure, with a redox potential of 277 ± 5 mV, at pH 7.0, that behaves as a monomer in solution. The globular Laz soluble domain adopts the Greek-key motif, with the copper center located at one end of the β-barrel coordinated by Gly48, His49, Cys113, His118 and Met122, in a distorted trigonal geometry. The edge of the His118 imidazole ring is water exposed, in a surface that is proposed to be involved in the interaction with its redox partners. The heterologously expressed Laz was shown to be a competent electron donor to *N. gonorrhoeae* cytochrome *c* peroxidase. This is an evidence for its involvement in the mechanism of protection against hydrogen peroxide generated by neighboring lactobacilli in the host environment.

1. Introduction

During infection, pathogenic microorganisms are frequently exposed to oxidative stress due to reactive oxygen species (ROS) generated by host defense mechanisms. Some of the most common ROS found in biological systems include superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO) [1]. However, there are other sources of ROS, such as the bacteria's own metabolic process, exposure to factors within host environment, such as metal ions, or commensal organisms that generate oxidants. Pathogenic bacteria from the species *Neisseria gonorrhoeae* and *Neisseria meningitidis*, that cause gonorrhea and meningitis, respectively, are constantly exposed to ROS from these various sources and, therefore, have developed numerous defense mechanisms to cope with oxidative stress [2]. These mechanisms are essential to cell survival, since ROS can damage DNA, proteins and cell membranes.

One of these defense mechanisms is based on cytochrome *c* peroxidases. These enzymes are *c*-type heme containing periplasmic enzymes that catalyze the reduction of hydrogen peroxide to water, using *c*-type cytochromes or small type 1 copper proteins, of the respiratory chain, as electron donors [3,4].

An example of such an electron donor is azurin, a member of a family of copper-containing, water soluble, low molecular weight redox proteins called cupredoxins, which function as electron shuttles in the electron transfer chain of several microorganisms, including some well-known pathogens, such as *Pseudomonas* (*Ps.*) *aeruginosa*. These proteins have received great attention due to their unusual spectroscopy and electronic structure in the oxidized copper(II) form, as well as, their ability to transfer electrons rapidly to several enzymes [5,6].

Ps. aeruginosa azurin has been shown to be involved in the protection against oxidative stress, since a mutant lacking this protein was shown to be very sensitive to ROS [1,7]. In another pathogenic bacterium, *N. gonorrhoeae*, a mutant strain lacking the gene coding for a cupredoxin, the lipid-modified azurin (Laz), was found to be much more sensitive to hydrogen peroxide but not to superoxide, unlike the *Ps. aeruginosa* azurin mutant, resulting in reduced survival in human ectocervical epithelial cells [2].

The *Neisseria* lipid-modified azurin has a high sequence homology to other copper proteins from the azurin family [8,9], though it differs significantly from other members of this family in that it contains a N-terminal domain of 39 amino acids that encodes the H.8 epitope (common to pathogenic *Neisseria*), in which there are five imperfect

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repeats of the sequence Ala-Ala-Glu-Ala-Pro (AAEAP) [9,10] (Fig. 1). In addition, it is modified by a lipid group (palmityl fatty acid) at its N-terminus [2,9,11], making Laz a lipoprotein bound to the outer membrane [10]. In fact, recently it has been shown using antibodies that *N. meningitidis* Laz is located at the outer membrane and that under certain growth conditions it can even be facing the outside of the cell [12]. Therefore, a similar location is expected for this protein in *N. gonorrhoeae* (tethered to the outer membrane and the facing periplasmic space).

The role of this electron shuttle protein is not completely established, since *Neisseria* genus has an array of periplasmic *c*-type cytochromes with apparent redundancy [13], shown to have complementary roles as electron shuttles in the respiratory chain of these microorganisms (Fig. 2). *Neisseria* genus can grow aerobically and microaerobically, as they present cytochrome cbb_3 oxidases with high affinity for oxygen, and can also use nitrite as an alternative electron acceptor, as they present an incomplete denitrification chain, composed by a copper nitrite reductase (AniA) and nitric oxide reductase NorB [14].

In *Ps. aeruginosa*, azurin can donate electrons to enzymes of the denitrification pathway, and thus, the role for *Neisseria* Laz as electron donor to AniA had been proposed, since these two proteins share the same cellular location, as being tethered to the outer membrane [10]. Nevertheless, in the case of *N. gonorrhoeae*, it was shown recently that the membrane bound cytochrome c_5 and CcoP are essential as electrons shuttles to AniA [13]. Therefore, the question remains as to the identity of the redox partner(s) of Laz in *N. gonorrhoeae*.

Based on primary sequence homology, Laz and azurin belong to the same cupredoxin family, the azurin-family [15]. These small redox proteins have distinct spectroscopic properties, with a strong absorption band at around 600 nm (with an ε of 3–6 mM^{-1} cm^{-1}), which has been assigned to a S_{cys}–Cu charge transfer band, and a redox potential higher than the one found in most inorganic copper complexes [16]. In cupredoxins, the copper atom adopts a distorted tetrahedral or bipyramidal geometry, with three of the four/five copper coordinating residues located at the C-terminus (Cys, His and Met) and the other(s) at the N-terminus. In the case of azurin, the fifth copper ligand is an oxygen from the carbonyl peptide bond of a glycine that is coordinating the copper atom, making the geometry more bipyramidal [15]. The structure of *N. gonorrhoeae* Laz incorporating Zn instead of Cu was recently determined by X-ray at 1.9 Å resolution (PDB ID 3AY2) [17]. In this work, we proceeded to solve the solution structure with Cu so as to evaluate any differences between Zn-Laz and the native like form especially since Zn(II) prefers a quadrangular planar geometry.

This work focuses also on the biochemical characterization of *N. gonorrhoeae* Laz, as well as, on the determination of the solution structure of its cupredoxin domain. Results obtained show that Laz can donate electrons to cytochrome *c* peroxidase from the same organism, which might explain the involvement of this cupredoxin in the defense against oxidative stress.

2. Materials and methods

2.1. Protein purification

The gene encoding the soluble part of Laz was cloned into a pET vector and the protein was heterologously produced in *Escherichia coli* BL21(DE3), as reported in [18]. The transformed strained was grown in M9 medium [18], to label the protein with ^{15}N or $^{13}C/^{15}N$ and in LB medium supplemented with 0.5 mM $CuSO_4$, for the biochemical characterization. The same induction conditions were used in both cases and the protein was purified in two chromatographic steps [18], an anionic exchange followed by a size-exclusion chromatography.

The purified Laz had a ratio of $A_{625\ nm}/A_{278\ nm}$ above 0.8, when completely oxidized. The Laz preparations were considered to be pure as judged by Coomassie Blue stained SDS and native PAGE. The protein was stored at −80 °C until further use.

2.2. Biochemical and spectroscopic characterization

2.2.1. Determination of the extinction coefficient

Copper (I) content was determined using a modified version of the method of Hanna et al. [19], which is based on the formation of a complex between Cu^I and 2, 2′-biquinoline in an acetic acid medium. All solutions were prepared fresh in deionized water. A sample of Laz (100 μL, containing 10–30 nmol of protein) or a standard solution of copper acetate was reduced by adding 300 μL of 20 mM sodium ascorbate (in 0.1 M sodium phosphate at pH 6.0) and incubated for 30 min. To this, 600 μL of a 2,2′-biquinoline solution (0.5 mg/mL) prepared in glacial acetic acid was added, and the solution was incubated for 10 min prior to the measurement of the absorbance at 546 nm. The concentration of Cu^I present in each sample was determined using the slope of the calibration curve prepared with copper(II) acetate. The extinction coefficient obtained (6.3 mM^{-1} cm^{-1}) was identical to that described by Hanna et al. [19]. The method used to quantify the total protein content was the BCA method (Sigma) [20], according to the manufacturer's instructions. The standard protein used was bovine serum albumin.

The extinction coefficient was determined taking into account the copper concentration of the sample, as a contamination with another copper protein is unlikely to occur, and it is rather difficult to have a pure protein sample (usually considered as pure when it is >95% purity).

2.2.2. Determination of molecular mass

The molecular mass of the purified Laz was determined by electrospray ionization mass spectrometry (ESI-MS). The apparent molecular mass of Laz was estimated by molecular-exclusion chromatography, using a Superdex 75 10/300 GL (GE Healthcare). The column was equilibrated with 50 mM Tris–HCl, pH 7.6 with or without 150 mM NaCl. Samples of oxidized Laz (1 nmol) were prepared in the running buffer. A calibration curve was prepared using the Gel Filtration Calibration Kit Low Molecular Weight (GE Healthcare) in the same running buffer, according to the manufacturer's instructions. The chromatograms together with the analysis of this data are shown as Supplementary material (Fig. S1 and Fig. S2).

2.2.3. Spectroscopic characterization

The visible-spectra were recorded on a Shimadzu UV-1800 spectrophotometer using 1 cm quartz cells. Laz samples were oxidized with a solution of potassium ferricyanide and reduced with a solution of sodium ascorbate.

The Laz sample for EPR was 0.3 mM in 20 mM phosphate buffer, pH 7.0. The EPR spectra of Laz were recorded on a X-band Bruker EMX

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1       10        20        30        40        50        60
MKAYLALISA AVIGLAACSQ EPAAPAAEAT PAGEAPASEA PAAEAAPADA AEAPAAGNCA
ATVESNDNMQ FNTKDIQVSK ACKEFTITLK HTGTQPKASM GHNLVIAKAE DMDGVFKDGV
GAADTDYVKP DDARVVAHTK LIGGGEESSL TLDPAKLADG DYKFACTFPG HGALMNGKVT
LVD
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Fig. 1. Analysis of the primary sequence of *Ng* Laz. In green underlined is the signal peptide recognized by the signal peptidase II pathway; in blue are identified the residues of the mature protein recognized by the epitope for the H.8 MAb; and in black the azurin-like domain.