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Biochemical Characterization of Bacterial Cytochrome c Peroxidase from the Human Pathogen Neisseria gonorrhoeae

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Abstract

*Neisseria gonorrhoeae* is an obligate human pathogen that expresses an array of molecular systems to detoxify reactive oxygen species as defense mechanisms during colonization and infection. One of these is the bacterial peroxidase that reduces H$_2$O$_2$ to water in its periplasm. The soluble form of this enzyme was heterologously expressed in *E. coli* in the holo-form binding two c-types hemes, a high-potential E heme and a low-potential P heme, with redox potentials of (+ 310 mV) and (- 190 mV / - 300 mV), respectively in the presence of calcium ions, at pH 7.5. Visible and EPR spectroscopic analysis together with activity assays indicates the presence of a calcium dependent reductive activation mechanism in the *Neisseria gonorrhoeae* bacterial peroxidase, in which P heme is 6cLS bis-His coordinated in the fully oxidized state of the enzyme, and becomes 5cHS upon reduction of E heme in the presence of calcium ions. The activated enzyme has a high affinity for H$_2$O$_2$ ($K_M$ of $4 \pm 1$ μM), with maximum activity being attained at pH 7.0 and 37 ºC, with the rate-limiting step in the catalytic cycle being the electron transfer between the two hemes. In this enzyme, dimer formation is not promoted at high ionic strength, thus differing from the classical bacterial peroxidases. These results contribute to the understanding of the involvement of *Neisseria gonorrhoeae* bacterial peroxidase has a first line defense mechanism against exogenously produced hydrogen peroxide in the host environment.

**Keywords:** *Neisseria gonorrhoeae*; bacterial peroxidase; heme enzyme; reactive oxygen species; kinetics.
1. Introduction

*Neisseria (N.) gonorrhoeae* is an obligate human pathogen that causes the sexually transmitted disease gonorrhea [1], that infects each year an estimate of 62 million people worldwide [2]. According to the World Health Organization (WHO reference number: WHO/RHR/11.14) a growing number of gonorrhea cases do not respond to the usual antibiotic treatment. The threat of these drug-resistant strains is increasing and new targets for gonorrhea treatment need to be assessed, such as, the bacteria defenses towards the host and neighboring microorganisms.

Pathogenic microorganisms, during infection, are frequently exposed to oxidative stress due to reactive oxygen species (ROS) generated by host defense mechanisms, such as hydrogen peroxide (H$_2$O$_2$) [3]. In order to cope with the oxidative stress, either endogenously or exogenously produced, *N. gonorrhoeae* has multiple enzymes that detoxify ROS but in particular, the main H$_2$O$_2$ scavengers are catalase (KatA) [4] and the bacterial peroxidase (*Ng*BCCP), encoded by the *ccp* gene [5], that catalyzes the reduction of hydrogen peroxide to water, and is the focus of this work.

In *N. gonorrhoeae*, the expression of *ccp* gene is induced when the bacteria is exposed to low oxygen tensions, as it is completely dependent on the anaerobic transcription factor FNR [6, 7]. It is well described that catalase has an important role in hydrogen peroxide detoxification, however, it is located in the cytoplasm. Therefore, *Ng*BCCP can be regarded as a first defense against exogenous hydrogen peroxide, by being located in the periplasm, which explains why a *ccp/katA* mutant has increased sensitivity to hydrogen peroxide in anaerobic conditions, in comparison to a *katA* mutant [8].

Previous biochemical studies on *Ng*BCCP indicated that it is a *c*-type diheme lipoprotein with a signal peptide cleaved by signal peptidase II [5] and attached to the outer membrane. Several soluble periplasmatic BCCPs have been isolated from various Gram negative bacterial species, with the first two described BCCPs purified from *Paracoccus (Pa.) pantotrophus* [9] and *Pseudomonas (Ps.) aeruginosa* [10]. However, no BCCP from a pathogenic organism has been fully characterized to date. In *N. gonorrhoeae*, it has been reported that this enzyme plays a crucial role during formation of biofilms and in the initial steps of infection [11], and thus a detailed study is justified.

Classical BCCP has two *c*-type hemes attached to the polypeptide chain through thioether bonds to two cysteines arranged in a -CXXCH- conserved motif and located in two different domains (Figure 1). There is a high-potential heme, in the C-terminal domain, His/Met
coordinated (6cLS/HS equilibrium at room temperature [12-15]), that acts as electron transfer center (E heme) to a low-potential heme, in the N-terminal domain, bis-His coordinated (6cLS) (in the fully oxidized state) (Scheme 1A), where the peroxidatic reaction will occur (P heme) [16-18]. The E heme in several BCCPs has a reduction potential, ranging from + 226 mV (Pa. pantotrophus BCCP [15]) to + 450 mV (Ni. europaea BCCP [19]), while P heme reduction potential ranges from - 150 mV, in Pa. pantotrophus BCCP [15], to - 330 mV in Ps. aeruginosa BCCP [18].

With the exception of Nitrosomonas (Ni.) europaea and Methyllococcus (M.) capsulatus BCCPs [19, 20], bacterial peroxidases are inactive in the fully oxidized state (Scheme 1A) and need reductive activation (Scheme 1B and 1C) [21]. This activation mechanism consists in several conformational changes in the polypeptide chain that occur after E heme reduction only in the presence of calcium ions [14, 17]. The most striking alteration is the removal of the distal axial histidine of P heme, which becomes a penta-coordinated high-spin heme (5cHS), and accessible to the substrate (Scheme 1B and 1C) [13, 22]. During this process there is formation of a π-stacking interaction between the aromatic side chain of W96 and the peptide bond of G95 of each monomer (Ps. aeruginosa BCCP numbering according to the primary sequence) [17], that contributes to the stabilization of the homodimer (Figure 1). These enzymes are described to exhibit a monomer-dimer equilibrium that is dependent on the presence of calcium ions, and promoted with increased ionic strength as the dimer interface has hydrophobic character [23, 24].

These enzymes typically use small redox proteins as electron donors, either c-type cytochromes [23, 24] or type 1 copper proteins (of the azurin or pseudoazurin sub-family) [25, 26], and in a few organisms more than one of these small electron shuttle proteins can act as electron donors [27]. Kinetic studies show turnover numbers ($k_{cat}$/[electron donor]) in the µM$^{-1}$.s$^{-1}$ range, as determined for different redox pairs, such as Shewanella (S). oneidensis BCCP/cytochrome $c_5$ (18 µM$^{-1}$.s$^{-1}$) [28]) and Rhodobacter (R.) capsulatus BCCP/cytochrome $c_2$ (2 µM$^{-1}$.s$^{-1}$) [29]. These turnover numbers are dependent on donor identity, binding
orientation, ionic strength and even pH, e.g. in *Pa. pantotrophus* BCCP/pseudoazurin the electron transfer rate constant is pH-independent between pH values of 5 to 9 [30]. In the absence of a known electron donor, artificial electron donors, such as, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS$^{2-}$) have been used to assess enzymatic activity. The turnover numbers achieved with saturating amounts of ABTS$^{2-}$ are in the same order of magnitude as the ones with other electron donors found in the literature [31, 32].

In the present study, *Ng*BCCP was heterologously produced in *E. coli* in a higher yield than previously reported, enabling its biochemical characterization. The specific catalytic activity was shown to be dependent on reductive activation and calcium ions, which also modulates its redox properties and monomer-dimer equilibrium. These studies show that *Ng*BCCP is a highly specific enzyme, with a unique P heme cavity with high and low-spin species observed in the mixed valence state at low temperature. This enzyme has proven to be an advantage for this pathogenic microorganism as a defense against the host immune response and could be a future therapeutic target to develop compounds against *N. gonorrhoeae*. 
2. Materials and Methods

2.1 Chemicals

Unless otherwise stated, all reagents were of analytical or higher grade and were purchased from Sigma-Aldrich and Fluka. Solutions were prepared in bi-distilled water or Milli-Q water when mentioned. EGTA solutions (ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid) were prepared by addition of 1 M NaOH to bring the pH to 8.0.

2.2 Bioinformatic analysis

All the analyzed protein sequences were obtained from the Protein database in NCBI (http://www.ncbi.nlm.nih.gov/protein) and the DNA sequences, that were translated, were deposited in the GenBank (http://www.ncbi.nlm.nih.gov/genbank). Multi-sequence alignments were performed using the software Mega6.06 [33] with ClustalW [34]. Jalview 2.9.0 software was used for sequence representation and consensus analysis [35]. A structural model of the globular domain of NgBCCP was created using SWISSMODEL [36] based on Ps. aeruginosa mixed valence BCCP structure (PDB:2VHD), as the primary sequence identity between these two enzymes is 49%.

2.3 Heterologous production of NgBCCP

NgBCCP encoding gene from N. gonorrhoeae FA 1090 was codon optimized for heterologous expression in Escherichia (E.) coli and synthesized in vitro. This sequence was cloned into an EcoRV digested pUC57 by NZYtech. The regions encoding the signal peptide and H8.epitope segments in the N-terminus of the protein were excluded, in order to obtain a soluble protein. The region of interest was amplified by PCR with primers that added a NeoI (Forward: CAATGCCATGGGCAGATCAGGACCTGCTGAAAC) and a XhoI (Reverse: CCAGCTCGAGTTGGTTGTCGCCGTTGCTTT) restriction sites, using pUC57 as template. This region was cloned into a pET22b (+) plasmid, named hereafter pET22-NgBCCP. The plasmid confers ampicillin resistance and adds a N-terminal signal peptide (pelB) to direct the encoding protein to the periplasm, and a C-terminal His-tag to aid in the purification. This cloning strategy introduced a Met-Gly, at the N-terminus after cleavage of the signal peptide, and a Leu-Glu-6His (His-Tag) at the C-terminus.

For plasmid propagation, an E. coli TOP10 strain (Invitrogen) was used. NgBCCP was produced in an E. coli BL21(DE3) strain (Novagen) co-transformed with pET22-NgBCCP
and pEC86 (harboring the ccm genes to produce the machinery for c-type heme biosynthesis and maturation [37], and confers chloramphenicol resistance).

Four to five colonies of the co-transformed E. coli BL21(DE3) were added to 50 mL of Luria-Bertani (LB) medium (10 g tryptone, 10 g NaCl and 5 g yeast extract, per liter) supplemented with 100 µg/mL ampicillin and 30 µg/mL chloramphenicol and grown overnight at 37 °C, 210 rpm. Fresh LB medium, supplemented with both antibiotics, was inoculated with 2% of pre-inoculum. Cultures were incubated under orbital shaking at 37 °C, 210 rpm until an O.D.₆₀₀nm of 1.5 was reached. At this point, cells were collected by centrifugation at 3500 g, 6 ºC, 20 min. The cell pellet was resuspended in half the volume of fresh LB medium with both antibiotics (adapted from Fernandes et al. [38]). In order to stabilize the cells they were incubated for 1 h at 37ºC, 120 rpm. NgBCCP expression was induced with 0.5 mM IPTG during 18 h at 30 ºC and 120 rpm. Finally the cells were harvested at 7500 g, 6 ºC, 10 min, and resuspended in 50 mM Tris-HCl, pH 7.6 containing protease inhibitors (cOmplete™, Mini, EDTA-free, Protease Inhibitor Cocktail Tablets, Roche).

2.4 Purification of Heterologous NgBCCP in E. coli

The periplasmic fraction was obtained by 5 freeze-thaw cycles and separated from spheroplasts and cell debris by centrifugation at 48000 g, 6 ºC, 15 min. Purification was performed in two chromatographic steps. The first step was an affinity chromatography using a 5 mL HisTrap column (GE Healthcare) equilibrated with 20 mM Tris-HCl, pH 7.6 and 500 mM NaCl. The periplasmic fraction was loaded into the HisTrap column and the unbound proteins were eluted with 5 column volumes of equilibration buffer, and an imidazole step gradient was applied from 0 to 500 mM, in equilibration buffer. NgBCCP was eluted with an imidazole concentration between 100-200 mM. The fractions containing NgBCCP were concentrated over a 5 kDa membrane VivaCell70 (Sartorius) and buffer was exchanged to 50 mM Tris-HCl, pH 7.6 with a desalting PD-10 column (GE-Healthcare) equilibrated in the same buffer. In the second purification step, this fraction was applied onto a gel filtration chromatographic column (HiLoad Superdex 200 16/600, GE Healthcare) equilibrated with 50 mM Tris-HCl, pH 7.6 and 150 mM NaCl. All fractions with A₄₀₂nm/A₂₇₈nm above 4.0 were considered pure and combined. The final NgBCCP fraction was concentrated and the buffer exchanged to 20 mM Hepes pH 7.5 using a desalting PD-10 column. A 12.5 % SDS-PAGE stained for protein (Coomassie blue) and heme content [9] was also used throughout the
purification to verify protein purity of the intermediate and final fractions. NgBCCP was stored in small aliquots at -80 °C until further use.

Protein and heme content was estimated using the modified Lowry method [39], with horse heart cytochrome c (Sigma) as standard protein, and pyridine hemochrome assay, respectively [40].

2.5 Biochemical characterization

2.5.1 Molecular mass determination

The apparent molecular mass of NgBCCP was estimated by molecular size-exclusion chromatography, with a Superdex 200 10/300 GL (GE Healthcare). The column was equilibrated with 50 mM Tris-HCl, pH 7.6, 150 mM NaCl with or without 2 mM CaCl₂, or 2 mM EGTA, to assess calcium ions dependence of apparent molecular weight. In order to assess the influence of ionic strength, the column was equilibrated with 50 mM Tris-HCl, pH 7.6, with or without 500 mM NaCl. Calibration curves were prepared using the Molecular Weight Gel Filtration Calibration Kit (GE Healthcare) according to the manufacture instructions, as well as a sample of NgBCCP in the as-isolated fully oxidized state, in the same running buffer. The chromatograms together with the analysis of this data are presented in the Supplementary Information (Figure S1).

The molecular mass of the purified NgBCCP was determined by electrospray ionization mass spectrometry (ESI-MS) on a Waters Synapt G1 HDMS mass spectrometer.

2.5.2 Spectroscopic characterization

The UV-visible spectra were recorded on a Shimadzu UV-1800 spectrophotometer, connected to a computer, using 1 cm-path quartz cuvette. The molar extinction coefficients were determined based on heme concentration, taking into consideration that there are 2 hemes/protein. NgBCCP in the mixed valence state (heme E reduced and P heme oxidized) was obtained by reduction with a solution of sodium ascorbate and diaminodurol (Asc/DAD) with a final concentration of 1 mM and 5 μM, respectively. In order to determine the effect of calcium ions in the visible spectrum of the mixed valence NgBCCP, a CaCl₂ solution was added to a final concentration of 1 mM.

The EPR spectra were recorded on a X-band Bruker EMX spectrometer equipped with a rectangular cavity (model ER 4102T) and an Oxford Instruments continuous liquid helium flow cryostat, at 10 K. The NgBCCP samples for EPR were 0.2 mM in 20 mM Hepes buffer,
pH 7.5. Samples were reduced with a solution of 1 mM sodium ascorbate/5 μM DAD, followed by addition of CaCl₂ solution to a final concentration of 2 mM. Experimental conditions for spectra acquisition were: 10 K, 20 mW of microwave power, 5 Gpp of modulation of amplitude, 1 x 10⁵ receiver gain and 3 scans were collected. Other experimental conditions are described in the figure legend of the spectra. The simulation of the spectra was performed using WINEPR SimFonia software version 1.2 (Bruker).

2.5.3 Potentiometric redox titrations
Potentiometric redox titrations of NgBCCP were performed under anoxic conditions (argon atmosphere inside a Mbraun anaerobic chamber), at room temperature, by measuring the absorption changes of a 8 μM NgBCCP (fully oxidized) in 100 mM Hepes, pH 7.5, and 2 μM of each mediator (reduction potentials for each mediator at pH 7.0 [41]: diaminoduroil + 260 mV, 1,2-naphthoquinone + 180 mV, phenazine methosulfate + 80 mV, phenazine ethosulfate + 55 mV, phenazine - 125 mV, 2-hydroxy-1,4-naphthoquinone -145 mV, flavin mononucleotide - 205 mV and neutral red -325 mV), with or without 2 mM CaCl₂, or with 2 mM EGTA. The reduction potential was monitored using a Pt pin electrode in combination with an Ag⁺/AgCl reference (Crison), and the potential, with reference to the standard hydrogen electrode, was obtained by adding 210 mV. Reductive titration was carried out by stepwise additions of small volumes of 0.1 - 100 mM sodium ascorbate, or 0.1 - 100 mM sodium dithionite, prepared in 100 mM Hepes, pH 7.5, while the oxidative titration was carried out by addition of 0.1 - 100 mM potassium ferricyanide. The spectra were scanned from 350 nm to 900 nm, using a TIDAS diode array spectrophotometer connected to an external computer. The absorbance at 554 nm was monitored and used to calculate a [oxidized]/[reduced] protein ratio for each acquired spectrum. This [ox]/[red] ratio was plotted as a function of the measured reduction potential at each titration point. The reduction potentials were then obtained from the simulation of the titration curves based on the Nernst equation of two independent redox centers.

2.5.4 Differential scanning calorimetry
In the differential scanning calorimetry (DSC) experiments, the NanoDSC instrument (TA Instruments) was loaded with degassed buffers (baselines and reference cell) and protein solution (20 μM NgBCCP in sample cell). Each protein sample passed through a desalting
NAP-5 column (GE Healthcare) equilibrated in the appropriate buffer (10 mM Hepes pH 7.5, with or without 2 mM CaCl$_2$, or 2 mM EGTA or 1 mM sodium ascorbate/5 µM DAD and 2 mM CaCl$_2$) and then diluted to the desirable concentration in the same buffer. The temperature was raised from 10 to 100 ºC at a scan rate of 1 ºC/min. The thermograms were analyzed with NanoAnalyze software from TA instruments using a non-two state model to simulate the data and obtain the melting temperature ($T_m$), the calorimetric ($\Delta H$) and van’t Hoff ($\Delta H_v$) enthalpies. The corresponding baseline was subtracted from each sample scan.

2.6 Kinetics with ABTS$^2^-$

The specific activity of NgBCCP using 2,2’-azino-di-(3-ethyl-benzthiazoline-6-sulphonic acid (ABTS)$^2^-$, from Sigma) as electron donor was determined by monitoring the increase in absorbance at 420 nm ($\epsilon_{420\text{nm}} = 36 \text{ mM}^{-1}\text{cm}^{-1}$) over time, as a result of ABTS$^2^-$ oxidation [42] in the presence of substrate H$_2$O$_2$, in an Agilent Diode Array. The assay was performed at 25 ºC in 10 mM Hepes pH 7.0, 10 mM NaCl and 1 mM CaCl$_2$, containing 3 mM ABTS$^2^-$, 100 µM H$_2$O$_2$, and initiated with 10 nM pre-activated NgBCCP (in all the assays, as otherwise stated). In the assay for the Michaelis-Menten curve, H$_2$O$_2$ concentration ranged between 0.01 and 1 mM. The temperature was varied from 15 ºC to 50 ºC, in order to assess the temperature dependence of the catalytic activity. The pH dependence assay was performed in the same conditions as above at distinct pH values, varying the buffer at a concentration of 10 mM, (MES buffer with pH values from 5.5 to 6.5, Hepes buffer with pH values from 6.5 to 8.0 and Bis-Tris-Propane buffer with pH values from 8.0 to 9.5).

The observed initial rates, $v_{\text{obs}}$, were determined in the first seconds of the re-oxidation curve, to which ABTS$^2^-$ auto-oxidation rates were subtracted to determine the real initial rates (initial velocity, $v_0 = \Delta[\text{ABTS}^2^-]\text{s}^{-1}$). Based on those rates it was possible to simulate a Michaelis-Menten curve to estimate $K_M$ and $V_{\text{max}}$. The turnover number, $k_{\text{cat}}$, given by $k_{\text{cat}} = V_{\text{max}}/[\text{NgBCCP}]$, was estimated using the enzyme concentration in the assay. The pH dependence of the catalytic activity was simulated using Equation 1, considering a di-acid-basic event:

$$v = \frac{v_{\text{high1}} + v_{\text{low1}} \cdot 10^{(pK_{a1}-pH)}}{1 + 10^{(pK_{a1}-pH)}} \cdot \frac{v_{\text{high2}} + v_{\text{low2}} \cdot 10^{(pK_{a2}-pH)}}{1 + 10^{(pK_{a2}-pH)}}$$

(1)

in which the initial rate, $v$, is given as a function of pH considering two $pK_a$ values. The values $v_{\text{high}}$ and $v_{\text{low}}$ are the average initial rate at high and low pH, respectively, for each acid-base equilibrium (for either $pK_{a1}$ or $pK_{a2}$). The amount of ABTS$^2^-$ consumed during the first 100 s as a function of the pH, was simulated using Equation 2: [43]
\[
\Delta[\text{ABTS}_{ox}] = \frac{\Delta[\text{ABTS}_{ox}]_{\text{max}}}{1 + 10^{(pK_a_1 - p\text{H})} + 10^{(p\text{H} - pK_a_2)}}
\]  

NgBCCP was pre-activated for all kinetic assays (as otherwise stated) in 5 \(\mu\)M enzyme stocks in 10 mM Hepes, 10 mM NaCl, pH 7.5, 0.2 mM sodium ascorbate, 5 \(\mu\)M DAD, 1 mM CaCl\(_2\), during 30 min, at room temperature, and then used directly in the assay.
3. Results and Discussion

3.1 Primary Sequence Analysis

*Neisseria* genus includes several species that colonize the human host, such as *N. polysaccharea*, *N. flavescens*, *N. sicca*, *N. cinerea*, *N. lactamica*, *N. subflava*, *N. elongata* subsp. *glycolytica*, *N. meningitidis* and *N. gonorrhoeae* [44], with only the last two being human pathogens. In the case of *N. meningitidis*, the *ccp* gene (encoding BCCP) was found in newly introduced genomes of strains that were obtained by whole-genome shotgun sequencing of isolates from over 1-year period from hospital's intensive care units [45]. The analysis of the primary sequence alignment of BCCP from these *Neisseria* species (not shown) indicates that they share a high percentage of identity (> 80 % identity), with most differences occurring in the N-terminal non-globular region.

In *NgBCCP* primary sequence are highlighted two specific features in its N-terminal (Figure 2) (i) the consensus cleavage site LAA'C recognized by signal peptidase II [5]. It is proposed that C21 becomes a diacylglycerolcysteine, that upon cleavage is N-acylated forming N-acyldiacylglycerolcysteine, anchoring the enzyme to the membrane [46]; and (ii) a low complexity linker region (LCR) (black box; imperfect repeats of AAEAP), rich in alanines, that enables the enzyme to have some flexibility within the periplasm and to be ≈ 50-90 Å from the surface of the inner leaflet of the outer membrane [47]. This feature is also present in other neisserial outer membrane attached proteins (lipid-modified azurin (LAz), outer membrane lipoprotein (Lip) and copper nitrite reductase (AniA) [5, 47, 48]).

The comparative multiple sequence alignment of *NgBCCP* with the one of BCCPs with known three-dimensional structure and of *M. capsulatus* BCCP is shown in Figure 2, with the conserved residues being displayed on the *NgBCCP* model structure in Figure 1B. This alignment indicates the presence of two c-type heme binding motifs, -CXXCH-, one at the N-terminal (P heme) and another in the C-terminal (E heme) region. The corresponding axial ligands of P heme (H115 and H131; *N. gonorrhoeae* FA 1090 BCCP numbering) and of E heme (H260 and M336) are conserved, as well as, the calcium binding residues (N139, T317 and P319) and the glutamate (E174) proposed to be involved in the catalytic mechanism, by promoting the cleavage of hydrogen peroxide at the O-O bond [49, 50]. Another conserved feature is the tryptophan residue, W154, that is proposed to be involved in the electron transfer pathway between the two hemes. This residue is located in the interface between the two domains, with its indole ring bridging the propionates of the two hemes (Figure 1B) [16].
Besides the conserved features, \( Ng \)BCCP presents a few differences in residues (conversed in the other analyzed sequences) located in loop/flexible regions which are involved in the structural changes between fully oxidized and mixed valence state (mainly in P heme domain), suggesting that this enzyme will have different biochemical properties.

The most significant difference is in the tryptophan residue that during activation forms a “Gly-Trp” stacking motif with a symmetry-related “Gly-Trp” pair in the neighboring monomer [17, 22] (W96 in \( Ps. \ aeruginosa \) BCCP sequence, see Figure 2) that is conserved in all BCCPs, with the exception of the one from \( N. \) gonorrhoeae and \( M. \) capsulatus. The formation of this stacking hydrophobic interaction is proposed to stabilize the distal His-ligand loop that moves away from the P heme, and consequently the dimer. In fact, all BCCPs have been described to be homodimers. This small but significant change might have led to small variations in the primary sequence, which are for instance observed in \( M. \) capsulatus BCCP. In this enzyme, Gln129 substituted a Ile/Val residue and Gly137 substituted a proline residue, with both changes occurring in the same loop as the distal histidine. The absence of this residue will be further discussed when analyzing the monomer-dimer equilibrium of \( Ng \)BCCP.

3.2 Heterologous production of \( N. \) gonorrhoeae BCCP

The PCR amplified DNA fragment inserted into pET22b expression vector encoded only the conserved globular region of \( Ng \)BCCP (starting at E59, see Figure 2), as the flexible region at the N-terminal could interfere with the stability of the enzyme. In addition, a His-tag was introduced at the C-terminal to facilitate the purification, since the amount of protein obtained is very low and a pure sample could not be obtained otherwise. This recombinant \( Ng \)BCCP (from now on designated as \( Ng \)BCCP) has 339 residues and an expected molecular mass of 38780.7 Da with two attached hemes (molecular mass of the polypeptide chain, 37547.4 Da, plus the molecular mass of two \( c \)-type hemes, 1233.3 Da).

\( Ng \)BCCP was isolated from \( E. \) coli periplasm and purified in two chromatographic steps, an affinity chromatography, followed by a size-exclusion chromatography. At the end, a pure enzyme fraction was obtained, as judged by its SDS-PAGE (Figure 4A) and PAGE (not shown) with a single band, which has an absorption ratio \( A_{402\text{nm}}/A_{278\text{nm}} \) of 4.2. This isolation procedure has an average yield of 8.4 mg of pure \( Ng \)BCCP, per liter of growth medium.
The heme/protein ratio of purified \( NgBCCP \) was 1.8 ± 0.4, which indicated that there are 2 \( \epsilon \)-type hemes per polypeptide chain, as expected. Mass spectrometry analysis confirmed that the two hemes are covalently bound to the polypeptide chain and that the sample is pure, since \( NgBCCP \) gives a single species with a molecular mass of 38780.7 ± 0.5 Da by ESI.

### 3.3 Monomer-dimer equilibrium - Effect of calcium ions

The monomer-dimer equilibrium was studied in solution by size-exclusion chromatography, indicating that the as-isolated \( NgBCCP \) is a monomer that does not show a tendency to dimerize at high ionic strength (50 ± 2 kDa at I=50 mM, 45 ± 2 kDa at I=200 mM, and 44 ± 2 kDa at I=550 mM), contrary to what has been observed in other bacterial peroxidases [23]. The effect of calcium ions on this equilibrium was also analyzed, and \( NgBCCP \) behaves as a monomer when treated with EGTA (42 ± 2 kDa), while in the presence of calcium ions, it has an apparent molecular weight of 68 ± 2 kDa, which is consistent with dimer formation in solution (see Figure S1 in Supplementary Information). However, this calcium effect was only observed at enzyme concentrations above 10 \( \mu \text{M} \) (data not shown).

As mentioned, contrary to \( NgBCCP \), all other bacterial peroxidases have been reported to exhibit a monomer-dimer equilibrium in solution that is promoted by increasing the ionic strength besides the presence of calcium ions [23, 24, 51]. The dependence of this equilibrium on the ionic strength was stated as an evidence for the hydrophobic nature of the dimer interface [22, 24]. The comparative analysis of a structural model of \( NgBCCP \) obtained using SWISSMODEL (see Figure S2 in Supplementary Information) shows that \( NgBCCP \) dimer interface is less hydrophobic than that of other bacterial peroxidases. This fact, together with the absence of the Trp residue (G133 in \( NgBCCP \)), proposed to contribute to the stabilization of the BCCP dimer, explains the absence of ionic strength dependence of the dimerization and the need for higher protein concentrations to observe dimer formation in the presence of calcium ions, when compared with other bacterial peroxidases.

Differential scanning calorimetry was used to assess the distinct soluble states of \( NgBCCP \) and study the influence of the calcium ions on those states. The thermograms obtained in the different conditions are presented in Figure 3, with the respective simulations.
In the presence of excess calcium ions, \( Ng \)BCCP shows an endothermic transition, with a \( Tm \) of 46.9 °C (\( \Delta H = 823.1 \text{ kJ/mol}, \Delta H_v = 752.0 \text{ kJ/mol} \)), that was simulated with an independent non-two state transition model (Figure 3, solid line), considering that \( Ng \)BCCP is in the dimeric state. The thermogram of as-isolated \( Ng \)BCCP is broad with a thermal transition at 48.6 °C (\( \Delta H = 344.5 \text{ kJ/mol}, \Delta H_v = 340.6 \text{ kJ/mol} \)) (Figure 3, dash-dot line). This broad peak can be attributed to the presence of a mixture of species, \( Ng \)BCCP with and without calcium ions, as well as, monomers and dimers, since all solution states will behave as having distinct molecular weights. To assess the effect of calcium ions, \( Ng \)BCCP was treated with 2 mM EGTA. The thermogram of this preparation has an even broader transition (Figure 3, dotted line) than the one of the fully oxidized enzyme. This transition was simulated considering that \( Ng \)BCCP is a monomer, with a broad thermal transition occurring at 47.9 °C, with a \( \Delta H = 248.3 \text{ kJ/mol} \). It was not possible to determine the van’t Hoff enthalpy, because the data cannot be simulated with a single model as there are several overlapping intermediate species, which could not be identified.

The thermal stability of mixed valence \( Ng \)BCCP in the presence of calcium ions was also assessed by DSC. The data show a shift in the endothermic transition to 55.6 °C, an increase of around 9 °C in comparison to the fully oxidized state (Figure 3, dashed line). The calorimetric enthalpy is of the same order of magnitude as the one of the as-isolated \( Ng \)BCCP in the presence of calcium ions (\( \Delta H = 735.9 \text{ kJ/mol} \)). It was observed a strong aggregation at higher temperatures, which caused a negative \( \Delta C_p \), resulting in higher molar heat capacity. Any model used in the simulation of the data suggested a larger \( \Delta H_v \) due to aggregation (\( \Delta H_v > \Delta H \)). This aggregation might have been caused by the presence of sodium ascorbate, but in its absence there was a mixture of oxidized and reduced species (data not shown), probably due to partial oxidation of \( Ng \)BCCP during sample preparation prior to the DSC experiments.

These results show that in the presence of calcium ions, the thermal transition of as-isolated \( Ng \)BCCP becomes narrower with a higher molar heat capacity. This data was simulated considering that the enzyme is a dimer, which is consistent with the dimerization observed in solution in the presence of calcium ions. A similar result was obtained by Pettigrew et al. [23], though in that study it was also observed a shift in the thermal transition towards higher values in the presence of calcium ions. The comparison of the calorimetric (\( \Delta H \)) and van’t Hoff (\( \Delta H_v \)) enthalpies shows that in any case, \( \Delta H_v < \Delta H \), reflecting intermediate states for both enzymes. On the other hand, in the presence of EGTA the enzyme is proposed to behave
only as a monomer consistent with the low molar heat capacity of the thermal transition, and with the monomeric form observed in solution, similarly to what was observed for Pa. pantotrophus BCCP treated with EGTA [23]. The broad transition observed in the absence of calcium ions can be explained considering that the two domains of NgBCCP behave independently. This leads to the existence of distinct conformations (states) that denature as independent populations, and not as a single state. A similar interpretation was made for apo-calmodulin, that has two globular lobes, for which the domains flexibility and different hydrophobicity leads to the separate unfolding of the C-terminal and N-terminal lobe [52], though in the case of NgBCCP this separation is not resolved.

3.4 Spectroscopic characterization

The UV-visible spectrum of the as-isolated fully oxidized NgBCCP (Scheme 1A) has the usual features of an oxidized c-type cytochrome, with a Soret band at 402 nm (extinction coefficient of 222 ± 8 mM⁻¹cm⁻¹) (Figure 4B). This spectrum has also a shoulder around 360 nm and an absorption band at 620 nm, characteristic of the presence of high-spin species. In the spectrum of the mixed valence state (Scheme 1B), the Soret band splits into two absorption bands, one corresponding to the reduced high-potential E heme, with a maximum at 418 nm, and the other to the low-potential P heme still in the oxidized state, as a shoulder of the E heme's Soret band with an apparent maximum absorption at 402 nm. A α- and β-bands, at 554 and 524 nm, respectively, become evident, consistent with E heme reduction.

The analysis of these spectra indicates that the as-isolated NgBCCP is fully oxidized and the observed high-spin species can be attributed to the E heme, that has been reported to be in a low/high-spin equilibrium, due to the loosely bound axial methionine ligand [12, 13]. In the mixed valence state (Scheme 1B), the high-spin band at 620 nm does not disappear completely indicative of the presence of a high-spin penta-coordinated heme, attributed to the P heme, as in other bacterial peroxidases, such as the ones isolated from Ps. aeruginosa and Ps. stutzeri [13, 24], that do not require additional calcium ions. Addition of calcium ions has only a small effect in the P heme's Soret band, with no additional increase in the absorption at 620 nm (Scheme 1C). Nevertheless, addition of EGTA to the mixed valence NgBCCP leads to a decrease in the high-spin absorption bands at 360 and 620 nm, together with an increase
in intensity of the absorption band at 402 nm (Soret band of P heme in the oxidize state, data not shown), which is an indication of the decrease in high-spin state of this heme (Scheme 1B). Therefore, this is consistent with high-spin formation at the P heme in the mixed valence state when calcium site is occupied.

In Figure 5 is presented the EPR spectra of NgBCCP in the as-isolated fully oxidized, mixed valence and mixed valence state incubated with calcium ions (the simulations of these spectra are presented in Figure S3 in Supplementary Information).

In the spectrum of the as-isolated fully oxidized NgBCCP (Figure 5A, Scheme 1A), two low-spin and a high-spin ferric species are observed. One of the low-spin species, with $g = 3.18$, is a Highly Axial Low Spin (HALS) signal, originated from the E heme with the other pair resonance not being detected in the EPR spectrum, as observed for other low-spin ferric heme proteins with a large $g_{\text{max}}$. The signals with $g_z = 2.99$ and $g_y = 2.25$ are assigned to a low-spin ferric species originated from P heme. According to the equation for low-spin hemes $g_z^2 + g_y^2 + g_x^2 = 16$, there should be another signal with $g_x = 1.41$, though it is most likely very broad and thus difficult to be observed. In this spectrum are also observed strong high-spin signals, with $g$ values of 6.28, 5.56 and 1.99. This agrees with the strong absorption band observed at 620 nm in the UV-visible spectrum of the fully oxidized enzyme, at room temperature. The observation of two high-spin signals (at 6.28 and 5.56) in the EPR spectra of bacterial peroxidases has also been reported for M. capsulatus Bath and Ps. stutzeri as-isolated bacterial peroxidases [20, 24].

- Insert Figure 5 -

The spectrum in Figure 5B, shows that after 1 min incubation with Asc/DAD (Scheme 1B), E heme is reduced and becomes EPR silent, which is supported by the absence of the signal at $g = 3.18$, the low-spin species of E heme, as well as, the disappearance of most of the $g_\parallel = 6.28$ and $g_\perp=1.99$-high-spin signal, that thus can be partially attributed to this heme. However, the high-spin species does not disappear completely, similarly to what was observed in the room temperature UV-visible spectrum (Figure 4B) and this new signal is attributed to P heme. A new set of $g$ values appear for a low-spin species, with $g_z = 2.88$, $g_y =2.36$ and $g_x =1.47$, substituting the previous signal with a $g_z = 2.99$ and $g_y = 2.25$. This change in $g$ values of P heme, with the signals becoming sharper, upon reduction of E heme, has been explained by the change in coordination sphere of this heme, from 6cLS to 5cHS (by loss of the distal
histidine ligand, Scheme 1B). However, at cryogenic temperatures, 5cHS species for P heme is not usually observed in the EPR spectrum [15, 55]. The presence of this low spin species has been attributed to P heme being coordinated with by a water molecule, as the sixth axial ligand in substitution of the distal histidine ligand, as observed in the X-ray structures of mixed valence bacterial peroxidases [49]. As explained by Echalier et al., although a water molecule is considered a weak ligand, the binding to P heme at cryogenic temperatures originates, in the EPR spectrum, signals with spectroscopic properties of a low-spin heme, which have been attributed to the higher energy of the Fe-water coordination [22].

The observation of P heme coordination sphere change upon reduction of E heme, without added calcium ions, is an indication that in NgBCCP the calcium site is almost fully occupied, enabling the conformational change of the polypeptide chain, similarly to what was observed for Ps. aeruginosa [17] and Ps. stutzeri bacterial peroxidases [24], and in agreement with the absorption spectra presented before (Figure 4B). After 30 min incubation with Asc/DAD (Figure 5C, Scheme 1B), these signals become even sharper and there is also a small decrease in the $g_\parallel = 6.28$ high-spin signal, indicating that after 1 min, the E heme was not fully reduced, and that conformational change at the P heme was also not complete.

After incubation with calcium ions (Figure 5D, Scheme 1C), the signal of the low-spin species becomes broader, which can be attributed to small changes in the P heme population, and/or the environment around this heme. In fact, addition of calcium ions promotes dimerization of NgBCCP (a single solution state is observed in the DSC experiments for the fully oxidized and mixed valence enzyme). Dimerization is expected to change the environment around the P heme, as in spite of primary sequence differences, it is expected that a loop close to P heme, including the P heme histidine axial ligand, will move to the dimer interface as observed in other classical bacterial peroxidases [17]. This conformational change will modify also the coordination geometry of P heme, and explains the decrease in the amount of high-spin species with increase of the low-spin species in the EPR spectra of mixed valence NgBCCP in the presence of calcium ions (see Figure S3 in Supplementary Information).

The presence of a high-spin species in the EPR spectra of mixed valence bacterial peroxidase incubated with calcium ions is unique to NgBCCP, which can be attributed to differences in the active site and in the dimer interface. Moreover, the spectroscopic data also shows that the calcium site is partially occupied, which can be explained by the nanomolar range binding
affinity ($K_D$ of (8.5±0.5) nM) determined for mixed valence $Ng$BCCP (Figure S4 in Supplementary Information), a high affinity when compared with the one of $Pa. pantotrophus$ bacterial peroxidase ($K_D$ of 2.6 $\mu$M) [51].

3.5 Redox titration
The oxidative and reductive titrations of $Ng$BCCP incubated with calcium ions or EGTA (to remove calcium ions) are presented in Figure 6. As expected, there are two reduction potentials, corresponding to a low- and a high-potential redox center, which are titrated separately. The titration curves were simulated based on the Nernst equation and the values are listed in Table 1, which also lists the potentials obtained for the redox titration of the as-isolated $Ng$BCCP (these experiments were performed with the same enzyme preparation). It should be noted that, although the midpoint potential of both hemes was determined and discussed, the oxidation-reduction equilibrium $Fe^{III}/Fe^{II}$ of P heme is not relevant $in$ $vivo$, since the catalytic mechanism of BCCP does not include the fully reduced state of the enzyme.

In the presence of added calcium ions, the E heme of $Ng$BCCP has a midpoint potential of approximately $+310 \pm 10$ mV, in both reductive and oxidative titrations (Figure 6A and Scheme 1), showing that the reduction of E heme is reversible without hysteresis, even though its reduction is associated with considerable conformational changes in the enzyme [17]. On the other hand, the P heme, upon E heme reduction, is easier to reduce than it is oxidized, as its midpoint potential in the reductive direction ($-190 \pm 10$ mV) is higher than in the oxidative direction ($-300 \pm 10$ mV), with a hysteresis being observed.

In contrast, in the calcium depleted $Ng$BCCP (Figure 6B and Scheme 1), E heme is clearly more difficult to be reduced, with a 150 mV decrease in its reduction potential (with a midpoint reduction potential of $+170 \pm 10$ mV), compared with the value in the presence of calcium ions. Likewise, the P heme in the reductive titration is also more difficult to be reduced, by around 60 mV, and the hysteresis effect on the oxidative titration is not as significant as the one observed before in the presence of calcium ions (Table 1). This lack of
hysteresis can be explained by the absence of conformational change around the P heme. Thus, in the absence of calcium ions, P heme remains hexa-coordinated and low-spin regardless of the oxidation state of E heme. In the as-isolated state, the reduction potential of E and P hemes are closer to the ones obtained for the enzyme in the presence of calcium ions, and the hysteresis in the reductive and oxidative titration of P heme is also more pronounced, when compared to the one in the absence of calcium ions. This strongly suggests, as previously discussed, that the as-isolated \( NgBCCP \) has the calcium site partially occupied.

### 3.6 Steady-state kinetics with ABTS\(^2\)

\( NgBCCP \) was assayed for its peroxidatic activity using ABTS\(^2\) as an artificial electron donor [42] (Figure 7). The enzyme without reductive activation, in the presence of calcium ions, has very little catalytic activity (Figure 7A), that can be attributed to a small population that is in the mixed valence state. This is a clear indication that \( NgBCCP \) requires reductive activation. Moreover, the presence of calcium ions in the pre-activation of \( NgBCCP \) and in the kinetic assay is essential to attain maximum catalytic activity (see Figure S5 in Supplementary Information), thus calcium ions are required to promote complete conformational change around the P heme and maintenance of the active state. Spectroscopic data indicated that this conformational change occurs almost completely without excess of calcium ions, as little change was observed in the P heme high-spin species after addition of calcium ions to the mixed valence \( NgBCCP \) and the reduction potentials of as-isolated \( NgBCCP \) are closer to the ones of the enzyme in the presence of calcium ions. Therefore, the fundamental role of calcium ions for catalytic activity can be explained by its loss as a consequence of the dilution effect in the kinetic assay (5 \( \mu \)M stock to 10 nM in the assay), as was suggested by the observation that \( NgBCCP \) behaves as a monomer in the presence of calcium ions at concentrations bellow 10 \( \mu \)M. The loss of calcium ions due to dilution has been proposed by Pettigrew and co-workers [56], and also explained the results obtained for a monomeric mutant of \( S.\ oneidensis \) bacterial peroxidase [57].

- Insert Figure 7 -

The kinetic parameters for the catalytic activity of pre-activated \( NgBCCP \) in the presence of calcium ions using ABTS\(^2\), as electron donor, were a \( K_M \) of 4 ± 1 \( \mu \)M \( H_2O_2 \) and a \( V_{max} \) of
0.79 ± 0.05 µM ABTS\(^2-\cdot s^{-1}\) at pH 7.0 and 25 °C (in comparison with a \(K_M = 27 ± 14\) µM \(H_2O_2\) and a \(V_{max} = 0.019 ± 0.002\) µM ABTS\(^2-\cdot s^{-1}\) without reductive activation) (Figure 7A and 7B). The \(K_M\) for the mixed valence \(NgBCCP\) is similar to \(Geobacter sulferreducens\) CcpA, with \(K_M\) of 6.2 µM [31], using ABTS\(^2-\) in the same concentration as in the present study. Other bacterial peroxidases have \(K_M\) values in the same order of magnitude [24, 28, 50], which points out that \(NgBCCP\) is an efficient hydrogen peroxide detoxifying enzyme.

The \(k_{cat}\) of 79 s\(^{-1}\) obtained for \(NgBCCP\) is similar to the one of \(S. oneidensis\) tag free BCCP in the mixed valence state using \(S. oneidensis\) cytochrome \(c_5\) as electron donor [28]. However, in that study, the electron donor is not in saturating concentrations, as well as in most of the other reported kinetic studies of bacterial peroxidases.

Regarding the pH dependence, the highest catalytic rate was observed at pH 5.5 (Figure 7C), with a p\(Ka_1 = 5.9 ± 0.1\) and a p\(Ka_2 = 8.4 ± 0.1\). However, if one analyses the amount of substrate consumed after 100 s of reaction, by determining the concentration of oxidized ABTS\(^2-\) present at that time-point, the curve has a bell-shape with an optimum pH around 7.0 and the same p\(Ka\) values (p\(Ka_1 = 5.9 ± 0.1\) and p\(Ka_2 = 8.4 ± 0.1\)) (Figure 7D). The decrease in product concentration at low and high pH is attributed to enzyme inactivation or protonation/deprotonation of catalytically relevant residues. At low pH values, ABTS\(^2-\) is an efficient electron donor (high initial rates) but the changes in the protonation state of the enzyme at these pHs do not favor the reaction. On the other hand, at high pH values two effects can be contributing to the decrease in activity, the lower efficiency of the electron donor and the deprotonation of important residues for catalysis (as the peroxidatic reaction requires protons).

The temperature dependence of \(NgBCCP\) was assessed and maximum activity was observed at 37 ºC (data not shown), which is consistent with \(N. gonorrhoeae\) lifestyle as a human pathogen. Arrhenius and Eyring plots (see Figure S6 in Supplementary Information) were used to further analyze the effect of the temperature in the reaction rate, which shows a linear dependence, with the exceptions of the temperatures above 37 ºC, due to enzyme denaturation. Based on Arrhenius equation, it was possible to determine the activation energy, \(\Delta E_A\), associated with this reaction, which is approximately 8 ± 1 kJ/mol, and the fit of the data to the Eyring equation to obtain \(\Delta H^\ddagger = 5 ± 1\) kJ/mol and \(\Delta S^\ddagger = -0.17 ± 0.03\) kJ/mol. Therefore, \(\Delta G^\ddagger\) at room temperature is 57 ± 1 kJ/mol, which is comparable to the one estimated based on the \(k_{cat}\) at 298 K (79 s\(^{-1}\), of 48.04 ± 0.03 kJ/mol. The low value of the estimated entropy change indicates that there is formation of an ordered enzyme-substrate complex. Thus, the rate-
limiting step of the turnover does not involve substrate binding or release, and can be the internal electron transfer from E heme to P heme.

The kinetics with ABTS\(^{2-}\) show that NgBCCP is an efficient peroxidase *in vitro*, reducing H\(_2\)O\(_2\) to H\(_2\)O, even using this artificial electron donor. However, as observed in Figure 7D, the maximum difference in ABTS\(^{2-}\) concentration in the assay during 100 s is 12.4 ± 0.6 µM ABTS\(^{2-}\) at pH 7.0, showing that the reaction is not complete (not all H\(_2\)O\(_2\) was consumed), even though ABTS\(^{2-}\) is in saturating concentrations. One plausible explanation is that ABTS\(^{2-}\) might not be able to maintain NgBCCP in the mixed valence and active state or the enzyme is inhibited over time by H\(_2\)O\(_2\) due to Fenton reactions.
4. Conclusions

NgBCCP is a highly conserved enzyme in *N. gonorrhoeae* strains, anchored to the outer membrane. To further characterize NgBCCP, a recombinant soluble form was heterologously produced and purified, as a di-heme c-type cytochrome. The dimerization of NgBCCP, unlike in other bacterial peroxidases, is not promoted by the increase in ionic strength and it behaves mainly as a monomer in the as-isolated state, at least, up to 10 μM concentrations. This fact can be explained by the lower hydrophobic character of the dimer interface, and might be related to its localization *in vivo*, as being anchored to the membrane, the dimer does not need to be as strong as for an enzyme that is freely diffusing in the periplasm. Nevertheless, it shares with the classical bacterial peroxidases a common feature of dimer promotion and stabilization by calcium ions.

Due to the high affinity of the calcium site (*K*$_D$ in the nanomolar range), this site is practically occupied after purification (though its occupancy differs between preparations), as demonstrated by the redox and spectroscopic properties of the as-isolated NgBCCP. Calcium site occupancy together with reduction of E heme play a key role in the activation of the enzyme, which becomes more thermostable (with an increase of 9 ºC in the *Tm* of the mixed valence state). This activation mechanism involves conformational changes that lead to the modification of P heme coordination from 6cLS to 5cHS. One unique spectroscopic feature of mixed valence NgBCCP is the observation at cryogenic temperatures of a high spin species for P heme. This species can be a non-water coordinated P heme, which has not been reported in the literature for any other mixed valence bacterial peroxidase, which might reflect changes in the P heme cavity. These differences will be assessed when the X-ray structure of NgBCCP becomes available.

The calcium effect on monomer-dimer equilibrium, spectroscopic properties, catalytic activity and stability of NgBCCP, clearly indicate that the activation mechanism involves both the conformational change that leads to the modification of P heme coordination, which becomes available for substrate binding, and enzyme dimerization. In the *in vitro* studies, these two events are separated, which might not occur *in vivo* as calcium levels in the periplasm are within micromolar range (in human mucosa calcium levels reach millimolar concentrations) [58, 59]. Moreover, NgBCCP tethering to the outer membrane can foresee a higher local enzyme concentration and thus promote dimerization, as mentioned.

NgBCCP has a high affinity for its substrate (low *K*_M), and the optimum conditions are close to the physiological for *N. gonorrhoeae* (pH from 6.0 to 7.5 and a temperature of 37 ºC, as
being a human pathogen) indicating that this enzyme is well adapted to its host environment. Moreover, we propose that the limiting step in the catalytic cycle is the electron transfer from E to P heme.

In conclusion, the biochemical properties described here for NgBCCP indicate that this enzyme has a relevant role in the detoxification of hydrogen peroxide that N. gonorrhoeae encounters during proliferation and infection. Further studies are clearly necessary to assess this role, specially the interaction with the identified competent electron donor, the lipid-modified azurin NgBCCP [48] and also its tri-dimensional structure. Moreover, the fact, that NgBCCP is in an accessible location and that this enzyme is well-conserved in N. gonorrhoeae strains, but absent from human cells makes it a good candidate to design active compounds and also to use it as a target for immunization against these bacteria.

List of abbreviations
6cHS/LS - 6-coordinated in high-spin low-spin equilibrium
6cLS - 6-coordinated low-spin
5cHS - 5-coordinated high-spin

Author Contributions
CSN cloned and purified the NgBCCP, and performed and analyzed the spectroscopic data, redox titrations and DSC experiments, as well as, the bioinformatic analysis and model structure of NgBCCP. She has also analyzed the kinetic data and wrote the manuscript. MR performed the kinetic experiments and, GVD and BD performed the mass spectrometry experiments. SRP designed and planed the project and experiments, contributed to the analysis of the data and wrote the manuscript.

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References

Tables

Table 1 – Reduction potential of each NgBCCP heme in the reductive and oxidative direction in the presence of excess, without added (as-isolated) and in the absence of calcium ions (+ EGTA), at pH 7.5.

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<td>Oxidation</td>
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<tr>
<td>P heme</td>
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Figure legends

Figure 1 - (A) Schematic representation of NgBCCP dimer showing the relative arrangement of the hemes in the monomer and in the dimer. (B) Model structure of NgBCCP in the active state, displaying the two hemes, their axial ligands, and other conserved residues involved in calcium binding, electron transfer or catalysis. This was obtained as described in Materials and Methods.

Figure 2 – Multiple sequence alignment of bacterial cytochrome c peroxidases primary sequence. N. gonorrhoeae FA 1090 (Ng; GI: 59802088), M. capsulatus (Mc; GI:53756268), S. oneidensis (So; GI:24373735), Ps. aeruginosa (Psa; GI:15599783), Ni. europaea (Ne; GI: 30180613), Marinobacter hydrocarbonoclasticus (Mah; GI:56541647), Pa. pantotrophus (Pap; GI:916812477), R. capsulatus (Re; GI:940625801) and Geobacter sulfurreducens (Gs; GI:259090277). The coloring is in accordance with percentage of identity at each position, from darker color box (100 % identity) to white box (≤ 20 % identity). The sequences are sorted by pairwise identity to NgBCCP. The outlined grey box marks the NgBCCP peptidase II cutting site and the black box the low complexity region with imperfect sequence repeats, only present in this protein. The loops and flexible regions involved in reductive activation are outlined by dashed black boxes. Some residues are marked as: (●) axial ligands, (*) calcium binding residues and (▲) tryptophan at the dimer interface.

Figure 3 – Differential scanning calorimetry of NgBCCP in different redox conditions and effect of calcium ions. NgBCCP (20 µM) equilibrated in 10 mM Hepes pH 7.5 (dash-dot line), 10 mM Hepes pH 7.5 and 2 mM CaCl₂ (solid line), 10 mM Hepes pH 7.5 and 2 mM EGTA (dotted line), and 10 mM Hepes pH 7.5, 2 mM CaCl₂, 1 mM sodium ascorbate and 5 µM
DAD (dashed line). The thermograms were baseline corrected and normalized for concentration. The simulations fitting the data are represented by grey lines.

**Figure 4** – UV-visible spectra of heterologously produced NgBCCP. (A) Coomassie blue stained SDS-PAGE of purified NgBCCP in a 12.5% Tris-Tricine gel (Lane 1 - Protein Marker; Lane 2 - NgBCCP). (B) UV-visible absorption spectra of NgBCCP, in 10 mM Hepes pH 7.5. The spectrum of the as-isolated NgBCCP is displayed as a solid line, the dashed and dotted lines are the spectra of the mixed valence and mixed valence incubated with calcium ions for 10 min, respectively. The inset shows the high-spin region of the visible spectra.

**Figure 5** – X-band EPR spectra of NgBCCP in different redox conditions and effect of calcium ions. A) 0.2 mM NgBCCP in the fully oxidized state, (B) after 1 min incubation with Asc/DAD, (C) after 30 min incubation with Asc/DAD and (D) after 30 min incubation with 2 mM CaCl₂ after reduction with Asc/DAD. The asterisk (*) indicates the presence of a small fraction of free iron. The low-spin region of the spectra was amplified 3 times in all cases.

**Figure 6** – Effect of calcium ions in the potentiometric titrations of NgBCCP at pH 7.5. The reduction potentials were determined in the presence of excess of calcium ions (A) or in its absence, in the presence of EGTA (B). The reductive titration is represented by open circles and the oxidative titration by closed circles. The lines represent the simulation of the potentiometric curve using the reduction potentials listed in Table 1.

**Figure 7** – Steady-state kinetics using ABTS²⁻ as electron donor. Catalytic activity of NgBCCP without (A) and with pre-activation (B). pH effect on pre-activated NgBCCP catalytic activity (C) and, for each pH, the average amount of ABTS²⁻ consumed in a 100 s
interval starting after addition of the enzyme (D). The dashed lines represent the simulated data using Michaelis-Menten, and Equations 1 and 2 in Materials and Methods. The parameters used in the simulation are the ones stated in the main text.
Scheme Legends

Scheme 1 - Schematic representation of NgBCCP indicating the spin-state and coordination sphere of both heme in the as-isolated fully oxidized form (A), mixed valence form without calcium ions in solution (B) and with additional calcium ions in solution (C). (A) In the fully oxidized state, ferric E heme is in a 6cHS/LS equilibrium at room temperature and the ferric P heme is 6cLS. (B) In the mixed valence form, E heme is reduced and becomes low-spin. If the calcium binding site is occupied, the reduction of E heme causes conformational changes that result in the removal of the P heme axial histidine ligand, which becomes 5cHS (active form) and HS/LS at low temperatures. In both redox states there is a monomer/dimer equilibrium due to a fraction of unoccupied calcium binding sites. Addition of calcium ions (C) promotes full dimerization and simplification of solutions states, with P heme being as 5cHS, and same HS/LS observed at low temperatures.
Figures

Figure 1
Figure 2
Figure 3

![Graph showing excess Cp (kJ/mol.K) vs. Temperature (°C)]
Figure 4

A

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<td></td>
</tr>
<tr>
<td>25.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14.4</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

![Graph showing wavelength vs. absorbance](image-url)
Figure 5

- **A**: High Spin – E and P hemes
- **B**: Low Spin – E Heme
- **C**: Low Spin – P Heme
- **D**: Low Spin – P Heme

Magnetic field (G):
- 6.28
- 5.56
- 3.18
- 2.99
- 2.25
- 1.99
- 1.47

*Note: The peaks at 2.25 and 1.99 G are marked with an asterisk.*

- **X3A**, **X3B**, **X3C**, **X3D**: These denote peaks at specific fields.

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Figure 6

A

B
Figure 7

A

B

C

D

$V_0$ (µM ABTS/s) vs. $H_2O_2$ (µM)

$V_0$ (µM ABTS/s) vs. $H_2O_2$ (µM)

$V_0$ (µM ABTS/s) vs. pH

$\Delta[ABTS_0]$ (µM) vs. pH
Scheme

Scheme 1

A. As-isolated Form (Fully oxidized)

B. Mixed valence Form

C. Mixed valence Form + Ca^{2+} (fully active form)
Graphical Abstract

*Neisseria gonorrhoeae* bacterial peroxidase was heterologously produced in higher yield than previously reported, enabling its biochemical characterization. Specific activity is dependent on reductive activation and calcium, which also modulates redox properties and monomer-dimer equilibrium. This enzyme is highly specific and a first line defense mechanism against exogenously produced hydrogen peroxide.
Highlights

- Biochemical characterization of *Neisseria gonorrhoeae* bacterial peroxidase
- Calcium ions promote dimerization and are essential for activation and activity
- Reductive activation is required to attain maximum activity
- Optimum pH and temperature in the physiological range
- The catalysis rate-limiting step is the electron transfer between the two hemes