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Heme-iron utilization by *Leptospira interrogans* requires a heme oxygenase and a plastidic-type ferredoxin-NADP$^+$ reductase

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ABSTRACT

Background: Heme oxygenase catalyzes the conversion of heme to iron, carbon monoxide and biliverdin employing oxygen and reducing equivalents. This enzyme is essential for heme-iron utilization and contributes to virulence in *Leptospira interrogans*.
Methods: A phylogenetic analysis was performed using heme oxygenases sequences from different organisms including saprophytic and pathogenic *Leptospira* species. *L. interrogans* heme oxygenase (LepHO) was cloned, overexpressed and purified. The structural and enzymatic properties of LepHO were analyzed by UV–vis spectrophotometry and ^{1}H NMR. Heme-degrading activity, ferrous iron release and biliverdin production were studied with different redox partners.
Results: A plastidic type, high efficiently ferredoxin-NADP$^+$ reductase (LepFNR) provides the electrons for heme turnover by heme oxygenase in *L. interrogans*. This catalytic reaction does not require a ferredoxin. Moreover, LepFNR drives the heme degradation to completeness producing free iron and α-biliverdin as the final products. The phylogenetic divergence between heme oxygenases from saprophytic and pathogenic species supports the functional role of this enzyme in *L. interrogans* pathogenesis.
Conclusions: Heme-iron scavenging by LepHO in *L. interrogans* requires only LepFNR as redox partner. Thus, we report a new substrate of ferredoxin-NADP$^+$ reductases different to ferredoxin and flavodoxin, the only recognized protein substrates of this flavoenzyme to date. The results presented here uncover a fundamental step of heme degradation in *L. interrogans*.
General significance: Our findings contribute to understand the heme-iron utilization pathway in *Leptospira*. Since iron is required for pathogen survival and infectivity, heme degradation pathway may be relevant for therapeutic applications.

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1. Introduction

Iron is an essential nutrient for most organisms. Pathogenic microorganisms require host-iron for the metabolic processes that allow them to colonize their hosts and cause disease. Since free iron is not readily available and heme represents the most abundant source of iron in mammals, many pathogens have developed different mechanisms to acquire and utilize this molecule as iron source. A family of monooxygenases known as heme oxygenases (HO; EC 1.14.99.3) carries out heme degradation and enables microbes to use heme-iron [1,2].

HO, which employs heme as both substrate and a prosthetic group [3], catalyzes the conversion of heme to biliverdin IX, carbon monoxide (CO) and free iron (Fe^{2+}) utilizing oxygen and reducing equivalents [4].

HOs are present in a wide range of organisms including mammals [5], higher plants [6], algae [7,8], cyanobacteria [9], fungi [10], and various pathogenic microorganisms [11–15]. HOs play important roles in different physiological processes as iron homeostasis, defense against oxidative and cellular stress, neurotransmission by the generation of CO as a physiological messenger molecule [16] and biosynthesis of photoreceptive pigments in photosynthetic organisms. In pathogenic microorganisms the major function of HOs has been attributed to iron acquisition from host heme during infection [17], although some of them have been identified to protect against heme toxicity [1].

Several HOs structures are available [18–24]. HOs are classified as "all alpha" proteins. They fold into single compact multi-helical domains containing two structural repeats of 3-helical motif. The heme is sandwiched between two helices called proximal and distal, and is coordinated by a His side chain in the proximal side and by a water molecule in the distal side [25]. Heme degradation is a complex set of

Abbreviations: HO, heme oxygenase; LepHO, *Leptospira interrogans* heme oxygenase; apo-LepHO, apo form of *Leptospira interrogans* heme oxygenase; FNR, ferredoxin-NADP$^+$ reductase; LepFNR, *Leptospira interrogans* ferredoxin-NADP$^+$ reductase; Fd, ferredoxin; LFd2, *Leptospira interrogans* 2[4Fe-4S] ferredoxin; BV, biliverdin

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reactions that requires the input of several electrons originating from NADPH and three molecules of oxygen. While the mechanism of heme cleavage is broadly conserved between HOs from most organisms [2] (see above), the source of reducing equivalents is variable. In mammals the electrons required to drive the heme oxygenase reaction are derived from NADPH cytochrome P450 reductase [26]. Plant, cyanobacterial and bacterial heme oxygenase activity may be ferredoxin-dependent, as it has been suggested that the NADPH/ferredoxin-NADP(H) reductase/ferredoxin system may function to deliver electrons to these enzymes [27–29]. In some cases, the requirement of a second (auxiliary) reductant, such as ascorbate or trolox, has been proposed [28]. However, it was reported that the catalytic activity of *Pseudomonas aeruginosa* heme oxygenase requires only a ferredoxin-NADP$^+$ reductase [30].

Since iron is required for pathogen survival and infectivity, iron uptake and utilization is an attractive target for antimicrobial drug development [31]. Consequently, HO may be a potential target for new antimicrobials that hamper bacterial heme-iron utilization [32]. A knockout of the heme oxygenase gene in *P. aeruginosa* (pigA) shows severe growth defects [13]. Similar observations have been made with the pathogen *Leptospira interrogans* [14]. A mutant lacking the heme oxygenase gene (LB186) is growth impaired when hemoglobin is the only iron source in the medium, suggesting that HO is essential for heme-iron utilization by the spirochete. It has also been shown that *L. interrogans* HO contributes significantly to virulence in the hamster model of infection [33].

L. interrogans is a parasitic bacterium that infects humans and causes leptospirosis, also known as Weil's disease [34]. In the past few years this zoonotic disease has emerged as a major public health problem in much of the developing world. *Leptospira* also infects other mammals including rats, cattle, horses, pigs and dogs and some wild animals which are reservoir hosts of this pathogen. Leptospirosis is acquired via skin abrasions or the mucous membranes through contact with contaminated soil, water, or urine. *L. interrogans* colonizes the tissues of the host resulting in disease syndromes ranging from a mild flu-like illness to severe hemorrhagic disease.

The reaction catalyzed by heme oxygenase is dependent on reducing equivalents, since it requires NADPH and a reductase. Previously, we have proposed that this crucial function might be played by the ferredoxin-NADP(H) reductase (FNR; EC 1.18.1.2) in *L. interrogans* [35], as it has already been observed for the FPR enzyme of *P. aeruginosa* [30]. In this work, we studied the heme oxygenase from *L. interrogans* (LepHO). The location of LepHO gene and nearby open reading frames (ORFs) in the *L. interrogans* serovar Lai 56601 genome indicates that this locus participates in the acquisition and utilization of heme and probably in virulence. We also performed a phylogenetic analysis to investigate how this enzyme is related to other HOs. To infer the metabolic role of LepHO we studied its functional and structural properties. We found that the plastidic-type ferredoxin-NADP$^+$ reductase is able to efficiently support the catalytic activity of LepHO in vitro. Moreover, the reaction proceeds to completion producing biliverdin and free iron, without the need of a ferredoxin. These results suggest that the flavoenzyme is the redox partner of *L. interrogans* heme oxygenase in vivo.

2. Materials and methods

2.1. Sequence alignment and phylogeny

The HO amino acid sequence from *L. interrogans* NP_714730.1 (GI:24217247) was used as query sequence to perform standard tBLASTn through the complete protein and translated NCBI database (Release 195, Apr 2013). The phylogenetic analysis was performed using 107 sequences. After sequence alignment using ClustlX (2.0) [36] poorly aligned positions and divergent regions of the alignment were removed using the more conservative settings of the program Gblocks [37]. The extracted multiple alignment blocks were then used for obtaining a tree by the Bayesian inference method using the MrBayes package (v3.1.2) [38]. A fixed preset Whelan–Goldman model of amino acid substitution was used [39]. The Markov Chain Monte Carlo method was run with the following settings: 4 chains; temperature 0.25; 4,000,000 generations, sampling every 200 generations; and "burn-in" to discard the first 5,000 trees. Finally, a consensus tree was obtained (50% majority rule) and plotted using Dendroscope V 3.2.5 [40].

2.2. Construction of the LepHO expression vector

The gene encoding LepHO (LB186) was amplified by PCR using the genomic DNA from *L. interrogans* serovar Lai 56601, kindly provided by Dr Xiao-Kui Guo from the Dept. of Microbiology Shanghai Second Medical University, Shanghai, China. Oligonucleotides sequences 5'-cgcggatccatgagtttagcaactattttacg-3' and 5'-cccaagcttttaacctttttccaagaacggaatc-3' were designed to introduce *Bam*HI and *Hind*III restriction enzyme sites at the 5′ and 3′ ends, respectively. The expression plasmid was constructed by inserting the amplification product, previously cut with the indicated enzymes, into the similarly restricted pET-TEV vector, a modified pET28a vector that contains a Tobacco Etch Virus (TEV) protease cleavage site between the N-terminal hexahistidine (His6) tag and the multiple cloning site.

2.3. Protein expression and purification

The pET-TEV vector harboring the LepHO gene was transformed into *E. coli* BL21 (DE3) for protein expression. A single colony was cultured overnight in 10 ml of LB medium supplemented with kanamycin (50 μg/ml); the cell suspension was subsequently transferred to 1 L of fresh LB-kanamycin medium and grown at 37 °C until the OD_{600} reached 0.7–0.8. Protein expression was induced by addition of isopropyl 1-thiol-D-galactopyranose (IPTG) to a final concentration of 0.5 mM, and the culture was maintained during 16 h at 20 °C with mild agitation. *E. coli* cells were harvested by centrifugation, and disrupted by sonication in 50 mM Tris–HCl (pH 8), 100 mM NaCl, 1 mM benzamidine. The lysate was centrifuged, and the resulting supernatant was loaded onto a TALON-cobalt affinity chromatography column (Clonthech Laboratories) previously equilibrated with 50 mM Tris–HCl (pH 8), 100 mM NaCl. Following a washing step, the recombinant protein was eluted with the same buffer containing 150 mM imidazole. The His6-tag was removed from apo-LepHO by adding the recombinant TEV protease during the dialysis, and the proteins were further separated by a subsequent TALON-cobalt affinity chromatography procedure. TEV protease was obtained as described [41]. Apo-LepHO concentration was determined using the bicinchoninic acid method supplied in a Pierce BCA® protein assay kit (Thermo Scientific). The purified protein was stored at −80 °C until use.

The ferredoxin-NADP$^+$ reductase (LepFNR) and ferredoxin LB107 (LFd2) from *L. interrogans* were expressed and purified as described previously [35]. Protein concentrations were determined by spectroscopy using the published extinction coefficient for LepFNR ($\varepsilon_{459\ nm}$ = 9.5 $mM^{-1}\ cm^{-1}$) or employing the above mentioned bicinchoninic acid method, in the case of LFd2.

2.4. Reconstitution of LepHO-heme complex and binding affinity of heme

The LepHO-heme complex was prepared by adding small increments of hemin (Fluka, prepared in 5 mM NaOH) to the purified apo-LepHO and following the optical absorbance ratio at 280/403 nm. Once saturation was achieved, the unbound hemin was removed by size exclusion chromatography using Sephadex G50 and Superdex™ 75 10/300 GL columns pre-equilibrated with 50 mM Tris–HCl (pH 8), 100 mM NaCl.

The incorporation of heme to apo-LepHO was monitored spectrophotometrically. Once the ferric heme complex is formed it gives a characteristic spectrum with a Soret absorbance peak at 403 nm that is highly distinguishable from the free heme spectrum (Soret band at 385 nm). Heme binding assay to apo-LepHO was performed by addition

of increasing concentrations of hemin (0.01 to 0.15 μM) while maintaining the enzyme concentration at 0.13 μM in 1 ml of 50 mM Tris–HCl (pH 8), 100 mM NaCl. After each addition, the spectrum between 300 and 800 nm was recorded, and the complex absorbance at 403 nm was calculated. A mathematical deconvolution was applied as follows: the absorbances of free hemin and LepHO-heme complex at 403 nm are proportional to their absorbances at 385 nm isosbestic point multiplied by a factor x and y respectively (Eqs. (1) and (2)). These values were calculated from a set of measurements at different concentrations of free hemin or LepHO-complex as $x = 0.85$ and $y = 1.94$.

$$A_{403}\,\text{free hemin} = xA_{385} \tag{1}$$

$$A_{403}\,\text{complex} = yA_{385} \tag{2}$$

During titrations the spectra of bound and unbound hemin are superimposed over the entire range of analysis. Therefore, to determine the dissociation constant of the LepHO-heme complex was necessary to differentiate and quantify the A_{403} of the complex from the A_{403} of free hemin (Eq. (3)).

$$A_{403}\,\text{total} = A_{403}\,\text{complex} + A_{403}\,\text{free hemin} \tag{3}$$

We then considered that the recorded absorbances at 403 nm (A_{403} total) were composed of two components: the A_{403} hemin multiplied by "a" and A_{403} LepHO-heme multiplied by "b" (Eq. (4)). The A_{403} hemin and A_{403} LepHO-heme are the estimated absorbances at that wavelength if all added hemin is free or in complex with LepHO respectively. The variables "a" and "b" are the fraction of free or bound hemin.

$$A_{403}\text{total} = aA_{403}\text{hemin} + bA_{403}\,\text{LepHO}-\text{heme} \tag{4}$$

Substituting Eqs. (1) and (2) in Eq. (4) and rearranging:

$$b = (A_{403}\,\text{total} - xA_{385})/(y-x)A_{385} \tag{5}$$

Consequently, the absorbance of the complex at 403 nm is obtained by Eq. (6):

$$A_{403}\,\text{complex} = byA_{385} \tag{6}$$

The calculated absorbance of the complex was then plotted against the hemin concentration to construct titration curves, from which the dissociation constant was obtained using a one site binding model fit.

2.5. Size exclusion chromatography

Gel filtration chromatography was performed on ÄKTA explorer system (Amersham Biosciences) equipped with a Superdex™ 75 10/300 GL size-exclusion column (GE Healthcare). Buffer containing 50 mM Tris–HCl (pH 8), 100 mM NaCl at a flow rate of 0.5 ml/min was used for equilibration of the column and eluent. Standards of known molecular weight were loaded to the column, and their elution volumes were determined spectroscopically at 280 nm. Apo-LepHO and LepHO-heme complex were loaded on the column and chromatographed using the same conditions as the molecular weight markers.

2.6. Determination of the extinction coefficient

The extinction coefficient at 403 nm ($\varepsilon_{403\ \text{nm}}$) for the LepHO-heme complex was determined by the pyridine hemochrome method [42].

2.7. NMR spectroscopy

NMR spectra were acquired on a Bruker Avance spectrometer operating at a frequency of 599.740 (^{1}H) MHz and referenced to the residual water peak at 4.7 ppm. Proton spectra were acquired with presaturation of the residual water peak over 10,000 data points, with a spectral width of 24 kHz, a 125 ms acquisition time, a 50 ms relaxation delay, and 20 k scans.

2.8. Spectrophotometric analysis of heme turnover by LepHO

Optical absorption spectra were obtained using a Shimadzu UV-2450 spectrophotometer, and all the measurements were carried out in 600 μl of 25 mM HEPES-KOH (pH 7.5) at 25 °C in a cell with 1 cm path length. Electron transfer reactions from NADPH to LepHO were evaluated using as redox protein partners LepFNR and LFd2. The standard assay contained 6 μM LepHO, 0.1 mg/ml catalase (Sigma), 3 mM glucose 6-phosphate, 300 μM $NADP^+$, 1 unit/ml glucose-6-phosphate dehydrogenase and 0.5 μM; 1 μM or 1.5 μM LepFNR. When the system FNR/Fd was evaluated, the final protein concentrations were 0.5 μM LepFNR/0.5 μM LFd2; 0.5 μM LepFNR/1 μM LFd2 or 1 μM LepFNR/0.5 μM LFd2. Reactions were started by addition of the NADPH reductase system. Spectral changes between 300 and 800 nm were monitored over a 30 min time period. Biliverdin formation was followed using the absorbance change at 680 nm.

Ferrous iron release during LepHO-catalyzed heme degradation was analyzed by addition of 250 μM ferrozine to the reaction medium. Time dependent spectral changes were monitored, and the absorbance at 562 nm was followed for formation of the ferrozine-ferrous iron complex.

When ascorbate was employed as reductant, 5 mM ascorbic acid was added to 6 μM LepHO, and the spectral changes between 300 and 800 nm were recorded for 70 min.

2.9. Kinetic parameters of the LepFNR-LepHO electron transfer reaction

For kinetic analysis 0.25 μM LepFNR was added to reactions containing 0.7 to 11.5 μM LepHO-heme, 0.1 mg/ml catalase and 300 μM NADPH. The decrease of the Soret peak at 403 nm was monitored during 200 s, and slopes determined by linear regressions 30 s after addition of the reductase were taken as the initial reaction rates. Enzyme activity (μmol LepHO reduced min^{-1} mg $LepFNR^{-1}$) was plotted against the concentration of LepHO, and a non-linear regression of the data was calculated to fit Michaelis–Menten kinetics.

Steady-state kinetic and binding data were fitted to the theoretical curves using SigmaPlot (Systat Software Inc., Point Richmond, CA).

2.10. HPLC analysis of LepHO reaction products

Upon completion of the LepFNR-mediated heme oxygenase reaction, glacial acetic acid (50 μl) and 5 M HCl (100 μl) were added to 500 μl aliquots of the assay mixture. The products were extracted into chloroform (500 μl), the organic layer was washed further with distilled water (3 × 500 μl) and reduced to dryness. The resultant residue was resuspended in 100 μL of 5% (v/v) HCl in methanol and esterified for 16 h at 4 °C. The products were diluted with distilled water (400 μl) and extracted into chloroform. The organic layer was then reduced to dryness and solubilized in 100 μl of mobile phase consisting of methanol:water (85:15, v/v). The sample was analyzed by HPLC on ÄKTA explorer system (Amersham Biosciences) equipped with a Hypersil™ ODS C18 column (3 μm, 150 × 4.6 mm, Thermo Scientific). The flow rate was 0.4 ml/min, and eluates were monitored at 380 nm [28]. The mixture of all four biliverdin IX isomers was synthesized by oxidative cleavage of hemin [43]. Hemin (5 mg) dissolved in 50% pyridine was treated with 0.1 M sodium phosphate buffer (pH 7.0) containing 50 mg of ascorbate and incubated at 37 °C for 5 h. The reaction mixture was then acidified and esterified as described above. The same treatment was carried out with a biliverdin hydrochloride standard (Santa Cruz Biotechnology). Additionally, a liquid chromatography-tandem mass spectrometry analysis was performed using an Agilent 1200 HPLC system, coupled to a

G1314C VWD UV detector and a micrOTOF mass spectrometer (Bruker Daltonik, Bremen, Germany) operating in the positive-ion mode.

3. Results

3.1. Genome localization, primary sequence analysis and molecular phylogeny of LepHO

The locations of LepHO gene and nearby ORFs in the *L. interrogans* serovar Lai 56601 genome [44] are shown in Fig. 1. LepHO (LB186) is coded in the forward strand of a region between position ~176,600 and ~187,800 of chromosome II. Overlapping the 3′ end of the LepHO gene is ORF LB187, which codes for a putative protein similar to those of the major facilitator transporter superfamily. These proteins constitute one of the largest groups of membrane transporters found in many bacterial species [45]. The other six ORFs in the region are coded in the reverse (minus) strand. ORF LB191 codes for an iron-regulated heme binding protein known as HbpA. This protein functions as a TonB-dependent receptor responsible for mediating acquisition of heme-containing molecules and was found only in some pathogenic *Leptospira* serovars [46]. The ORF LB183 codes for one of the four FUR homologues, regulatory proteins responsible for modulating the expression of genes involved in iron acquisition [47]. In the same region, we found ORF LB181 which codes for a putative membrane protein related to dolichyl-phosphate-mannose-protein mannosyltransferase. Although O-mannosylation was thought to be restricted to fungi, in recent years it has become clear that the activity is conserved in all kingdoms and that in bacteria it may contribute to evade the defense mechanisms of the infected host [48]. LB190 codes for a homolog to YbaB/EbfC DNA-binding family proteins. This family may play a role in DNA replication-recovery following DNA damage, but the function(s) of these proteins remains to be determined [49]. Finally, ORF LB192 is related to the protein HmuY, a heme-binding protein that retrieves heme from the host and then delivers it to outer-membrane transporters, the TonB-dependent receptor [50]. Clearly, LepHO is located in a genome locus that participates in the acquisition and utilization of heme, which is likely related to infection and host persistence.

To search for HO family members, the protein and translated databases available at NCBI were examined by BLAST [51]. Using the amino acid sequence from *L. interrogans* HO as query, sequences were retrieved and 107 manually selected (Table S1). The sequences were aligned using ClustalX2 [36] as described in experimental procedures, and relevant blocks were extracted from the multiple sequences alignment for phylogeny (Fig. S1). LepHO sequence contains a homologous His relevant for heme-iron coordination at position 15, as observed in many HOs [52]. Other amino acids reported to be involved in heme-protein interactions are completely conserved; these include Tyr 124, Gly 128 and 133, Lys 167 and Arg 171. The Gly rich region from residue 127 to 134, which is important for substrate (heme) entry and product (biliverdin) release, is also totally conserved in the LepHO (numbers as in LepHO, conserved residues are highlighted in red in Fig. S1).

We performed a Bayesian inference of a tree for the 107 HO sequences using a Markov Chain Monte Carlo approach, with Bayesian posterior probabilities of 60% or more. Results are shown in Fig. 2 in the form of an unrooted tree. Our analysis clearly displays well-defined groups of eukaryotes and prokaryotes. Major bacterial groups were properly clustered in monophyletic lineages. The two *Firmicutes* sequences included in the analysis were clustered together distantly from all other sequences. Other *Firmicutes* were highly divergent and therefore not included in the analysis. Likewise, during tree inference, we found that isoforms from land-plants were rather distant from all other HOs analyzed, as was previously observed [53,54]. It was therefore not possible to obtain convergence in our phylogenetic tree inference when plant HO sequences were included. Consequently, these HOs were omitted from our study.

As was previously found for other *Leptospira* proteins [35], *Spirochaetes* HOs share a common ancestor with their homologues from *Cyanobacteria*. However, it is evident that both groups diversified very early. Interestingly, the *Eukaryotes* HOs are closer to *Cyanobacteria* than other bacterial sequences, suggesting a monophyletic origin from a unique ancestor. HOs from *Cyanobacteria* and *Eukaryotes* containing two HO isoforms were segregated differentially.

Spirochaetes are all included in a well-defined group from a common ancestor. The saprophytes *Leptospira biflexa* and *Leptospira meyeri* are more closely related to each other than to pathogenic *Leptospira*. Interestingly, all saprophyte HO sequences have three residue insertion ($Q_{86}K_{87}K_{88}$) not observed in other HOs. All pathogenic *Leptospira* HOs clustered in a closely related group. *Leptospira liceraseace*, which has been defined as opportunistic or intermediate, is placed apart between the saprophytes and pathogenic *Spirochaetes*. This distinct separation of the different *Leptospira* HO orthologes may suggest a possible functional role in pathogenesis.

3.2. Overexpression and purification of LepHO

LepHO overexpression in *E. coli* BL21(DE3) causes a bright green color pigmentation of the cells due to biliverdin accumulation (data not shown), as has been observed upon overexpression of other HOs [29,55]. The apo form of LepHO was entirely recovered from soluble fraction of cell lysate. The protein was purified using cobalt-affinity chromatography and then excised from the His6-tag by digestion with a recombinant TEV protease. The apo-LepHO was obtained in homogeneous form as judged by a single band in SDS PAGE, which corresponds to ~26 kDa, a value close to the molecular mass predicted from the amino acid sequence (Fig. 3A). Mass spectrometry analysis confirmed the LepHO molecular weight to be 26.3 kDa. Typical purifications produced ~80 mg of protein per liter of culture.

3.3. Reconstitution of LepHO-heme complex

HOs use heme both as a substrate and a prosthetic group, and can form a stable complex with heme [3]. To inquire whether LepHO could form such a complex, the purified apo-protein was incubated with hemin and the absorption spectrum recorded after removal of the unbound hemin by filtration on Sephadex G50. The electronic absorption spectrum of the LepHO-heme complex differs from that of

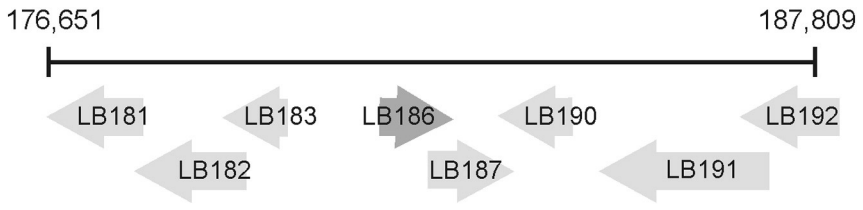

Fig. 1. Location of the gene encoding heme oxygenase (LB186) in the genome of *L. interrogans*. Genes that are close to HO coding sequence LB186 are shown as arrows with the corresponding identifier. See the text for details.

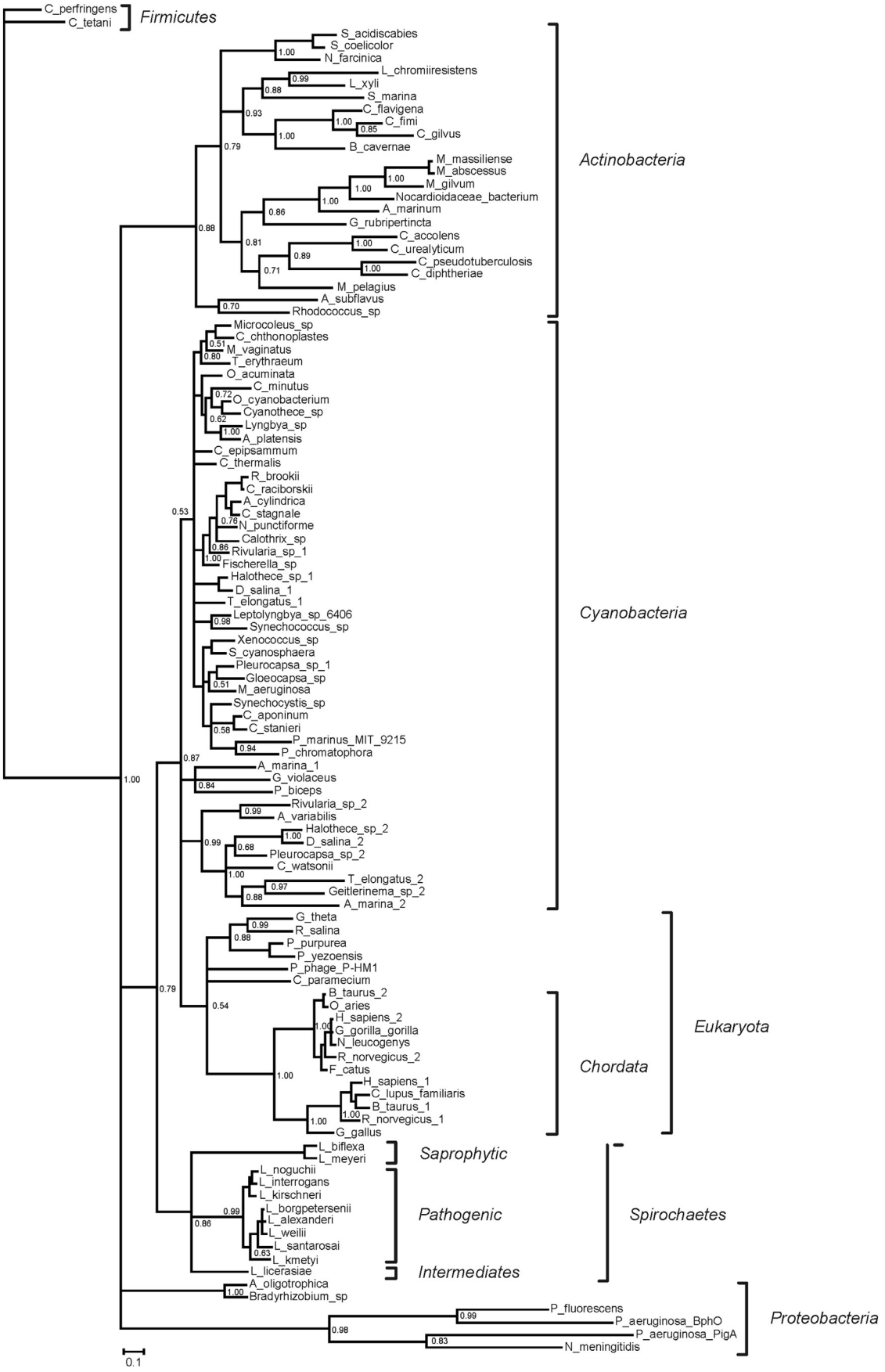

Fig. 2. Phylogenetic relationship between different heme oxygenases. The tree was constructed as described in Materials and methods. The distance between two sequences is represented as the lengths of the connecting branches. The database accession numbers and the full names of the HO source organisms are stated in the Table S1.

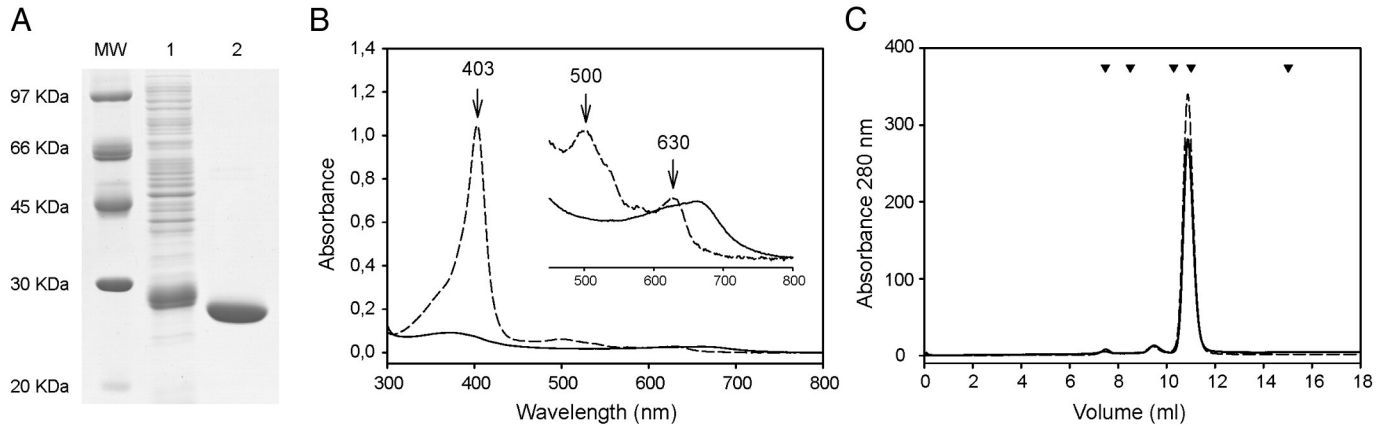

Fig. 3. Purification of apo-LepHO and its complex with heme. (A) SDS-PAGE analysis: lane MW, molecular weight markers; lane 1, soluble cell lysate of *E. coli* BL21 (DE3) expressing LepHO with the His6-tag; lane 2, purified apo-LepHO by cobalt affinity chromatography and subsequently digested with TEV protease. Numbers on the left indicate the molecular mass of the standards. (B) Optical absorption spectra of apo-LepHO (solid line) and its ferric heme complex (dashed line), which displays typical peaks at 403, 500 and 630 nm. The inset shows an enlargement of the region between 450 and 800 nm. (C) Gel-filtration chromatography profiles of apo-LepHO (solid line) and LepHO-heme complex (dashed line). The elution volumes of molecular mass standards are shown as triangles in the upper horizontal axe (in kDa: blue dextran, 2000; GST, 52; carbonic anhydrase, 29; GFP, 27 and aprotinin, 6.5).

the purified apo-LepHO (Fig. 3B). The ferric heme complex gives the characteristic spectrum of heme oxygenases, with a Soret band at 403 nm and peaks in the visible at 500 and 630 nm, which are comparable with those reported for bacterial HO-heme complexes [17]. The LepHO millimolar extinction coefficient at 403 nm was calculated to be 111.13 mM^{-1} cm^{-1}, a value that is close to those reported for other HOs [56].

The purified apo-LepHO and its ferric heme complex were subjected to gel permeation chromatography on a Superdex™ 75 column. Monitoring the absorbance at 280 nm (protein) confirmed that both samples elute mainly as single peaks corresponding to 28 kDa, thus indicating that the apo-protein and its heme complex are monomeric (Fig. 3C).

The incorporation of heme to apo-LepHO was determined by absorption spectrophotometry. Incremental addition of hemin to apo-LepHO allows visualization of the stoichiometric complex as reported for other HOs [57]. Since the spectra of heme and LepHO-heme complex overlap it was necessary to deconvolute the data as detailed in Materials and methods. Addition of hemin to a solution 0.13 μM apo-LepHO revealed a 1:1 stoichiometric relationship between protein and heme (Fig. 4). From these data a K_d of 0.017 ± 0.002 μM was estimated,

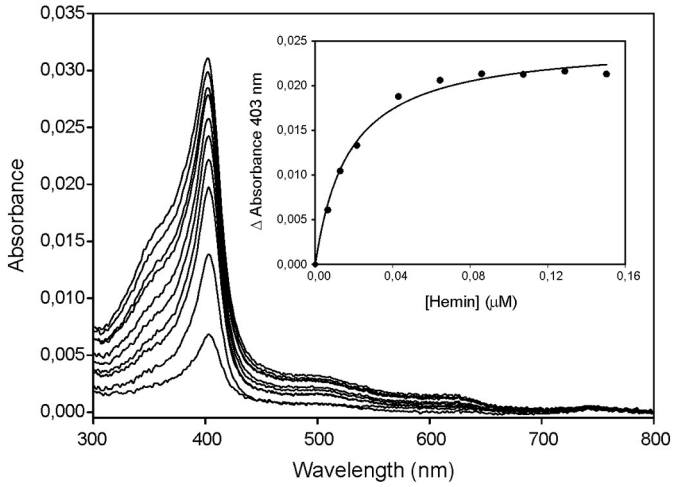

Fig. 4. LepHO-heme binding affinity. Optical absorption spectra of 0.13 μM apo-LepHO after addition of increasing amounts of hemin (0.01 to 0.15 μM). The inset shows LepHO-heme complex absorbance at 403 nm plotted as function of hemin concentration. The dissociation constant (K_d = 0.017 ± 0.002 μM) was obtained by fitting the data to a one site binding model.

a value lower than those previously determined for all known HOs [10,28,58].

3.4. NMR spectroscopic characterization of LepHO

It has been shown that the ^{1}H NMR spectra of cyanide-inhibited heme oxygenase (HO-CN) is a diagnostic tool of heme in-plane orientation, and hence regioselectivity of meso carbon oxidation [29,59]. The ^{1}H NMR spectra of α-biliverdin producing HO-CN enzymes are nearly identical and show only one heme methyl resonance (3 methyl, or 3Me) *ca.* 20 ppm in the paramagnetically shifted, downfield portion of the spectrum. Consequently, the fact that the ^{1}H NMR spectrum of LepHO-CN exhibits only the 3Me resonance *ca.* 20 ppm indicates that LepHO oxidizes heme to α-biliverdin (Fig. 5A). The presence of two methyl resonances originating from the 3-methyl group in LepHO-CN (3Me and 3me) indicates that heme binds in two orientations that differ by rotation about the α-γ-meso axis, as has been observed with human [60] and *Corynebacterium diphtheriae* HO [61,62]. The production of α-biliverdin by LepHO was further confirmed by HPLC analysis (Fig. 5B). The product extracted upon completion of the LepFNR-mediated heme oxygenase reaction showed a peak with a retention time of 15.1 min corresponding to the α-isomer of biliverdin standard.

3.5. Heme-degrading activity of LepHO

Ascorbate can serve as the electron donor in the oxidative degradation of heme by HO [63]. Therefore, the heme-degrading activity of LepHO was monitored by UV-visible spectroscopy after the addition of 5 mM ascorbic acid (data not shown). As the reaction proceeded, the LepHO Soret peak at 403 nm decreased, and a broad absorption band centered near 680 nm appeared, indicating that LepHO was able to cleave heme to produce biliverdin. As control, the same experiment in the absence of reductant did not shown any spectral change.

It is thought that cyanobacterial and eubacterial HOs are ferredoxin-dependent. However, it was stated that HO activity in cell extracts containing reduced ferredoxin also requires the presence of a second reductant, such as trolox or ascorbate [9,28,29,64]. In fact, in the absence of such auxiliary reducing agents, HO activity is very slow [9,64] or is arrested at the oxyferrous heme stage [29]. More recently, it has been observed that in *P. aeruginosa* a $NADP^+$-dependent ferredoxin reductase efficiently supports the catalytic activity of HO in vitro, without the need for a mediating ferredoxin [30]. In our case, incubation of LepHO with LepFNR and NADPH resulted in decreased Soret band intensity, with a concomitant shift to 410 nm and the appearance of two

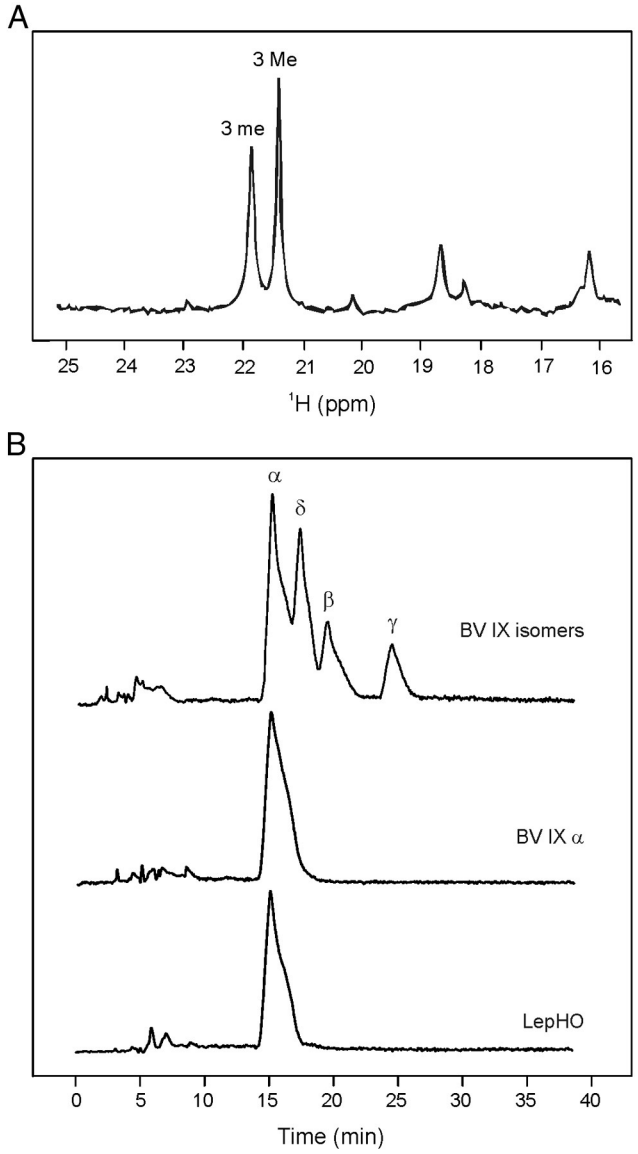

Fig. 5. Analysis of α-biliverdin production by LepHO. (A) Downfield portion of the ^{1}H NMR spectrum of cyanide inhibited LepHO obtained at 305 K from a 1 mM solution of LepHO-CN in 50 mM sodium phosphate buffer (pH 7.0). The presence of only one heme methyl resonance (3Me, or 3me) in this spectral region is diagnostic of α-meso carbon hydroxylating HOs. (B) LepHO reaction products were analyzed by HPLC after esterification as described in Material and methods. The absorbance of the eluate was monitored at 380 nm. BV IX isomers: mixture of the four biliverdin isomers obtained by chemical oxidative degradation of hemin; BV IXα: commercial α-biliverdin standard; LepHO: products extracted from LepFNR-LepHO reaction. Retention times for individual isomers were 15.1 min for BV IXα, 17.2 min for BV IXδ, 19.3 min for BV IXβ and 24.1 min for BV IXγ.

peaks with maxima at 538 and 575 nm (Fig. 6A). These spectral changes are consistent with the formation of a ferrous dioxyheme complex [Heme(Fe^{2+})-O_2] as has been previously observed [28]. Conversion of the latter to biliverdin is evident in the time-dependent decay of the 538 and 575 nm peaks and growth of a broad band at 680 nm. To establish that biliverdin was present in the reaction mixture a mass spectrometry positive-ion mode analysis was performed. Pigments extracted upon LepHO reaction showed a single high abundance peak with a mass-to-charge ratio of (1+) 583.25. In addition, the chemical formula of the molecule was predicted to be [$C_{33}H_{35}N_4O_6$]. These data were identical with the positive ion of authentic biliverdin standard. Taken together, obtained results indicate that LepFNR supports the LepHO catalyzed heme oxidation to biliverdin and free iron. In contrast, release of free iron and biliverdin in FPR/HO system from *P. aeruginosa*, requires the addition of acid or a chelating agent such as desferroxiamine [30]. To further characterize the LepFNR/LepHO reaction, activity measurements were performed using 0.25 μM LepFNR and increasing concentrations of LepHO (0.7–11.5 μM), monitoring the decrease in absorbance at 403 nm. Because the decay of Soret band intensity is the result of several reactions in the process of heme degradation, apparent Michaelis constants were estimated. Under the aforementioned conditions, a $V_{max\ app}$ value of 0.177 ± 0.007 μmol LepHO reduced min^{-1} mg $LepFNR^{-1}$ with a $K_{m\ app}$ for LepHO of 1.77 ± 0.21 μM were obtained.

To investigate the ferrous iron release by the NADPH/LepFNR/LepHO system the formation of an iron-ferrozine complex was monitored [65]. Reactions were performed as described before except that 250 μM ferrozine, a ferrous iron chelator, was added to the reaction media, and the spectral changes were followed during 45 min. As control, the same reaction was performed without LepFNR. Results from these experiments showed that addition of 0.5 μM LepFNR to the reaction mixture produced the typical disappearance of the Soret band at 403 nm and the simultaneous formation of a peak at 562 nm (Fig. 6B), demonstrating that under these conditions LepHO can release heme-iron. The production of 0.75 mol of ferrous iron per mol of heme after 45 min of reaction was determined using the extinction coefficient of the iron-ferrozine complex (27.9 mM^{-1} cm^{-1}) (Fig. 7C).

Heme-degrading activity of LepHO was also assayed with the *L. interrogans* FNR/Fd system. *L. interrogans* contains two different ferredoxins but only one of them, a 2[4Fe-4S] Fd (LFd2), can exchange electrons with LepFNR [35]. Initial measurements using 0.5 μM LepFNR showed a detectable increased rate of heme degradation by LepHO when 1 μM LFd2 was added (Fig. 7A). Although these results seem to suggest participation of LFd2 and LepFNR in relaying electrons from NADPH to LepHO, the dependency of LepFNR and LFd2 concentration on heme degradation was further analyzed at different LepFNR/LFd2 ratios. Results from these experiments showed that when LepFNR molar concentration is equivalent to that of the LepFNR/LFd2 system the rate of heme degradation by LepHO is similar (Fig. 7A). The same was observed when the reaction was monitored by following biliverdin formation (Fig. 7B). The increase of LepFNR concentration in the reaction medium overcame the effect caused by LFd2 addition (compare fill inverted triangles, which represent the 1.5 μM LepFNR condition, with open squares corresponding to 1 μM LepFNR/0.5 μM LFd2, in Fig. 7). Furthermore, when various LepFNR and LepFNR/LFd2 concentrations were analyzed different heme/Fe^{2+} conversion rates were obtained. However, final ferrous iron amounts produced were the same in all cases (Fig. 7C). These findings suggest that LepFNR efficiently supports the catalytic activity of LepHO in vitro, without the need of a ferredoxin, indicating that this flavoenzyme is the redox partner of LepHO in vivo.

4. Discussion

Iron acquisition by pathogenic bacteria is a critical step in the infection process and a central mechanism for host colonization. This creates a confrontation between the vertebrate defense mechanisms aimed at sequestering available iron and the pathogen strategies for scavenging the metal. The latter include the secretion of specific molecules, the participation of membrane transporters, sensors and regulators, and the involvement of metabolic systems for the extraction of iron from biomolecules [2,66].

One of the most important sources of iron in vertebrates is heme bound to proteins such as hemoglobin or myoglobin. HO in *Leptospira* has been reported as essential for host heme-iron utilization and for virulence [33]. The heme degradation reaction catalyzed by HO requires an efficient redox system to provide the necessary electrons. Hence, the main objectives of this work were to biochemically characterize LepHO, to analyze in depth the catalytic activity of heme degradation, and to elucidate the protein partner that delivers the necessary reducing equivalents.

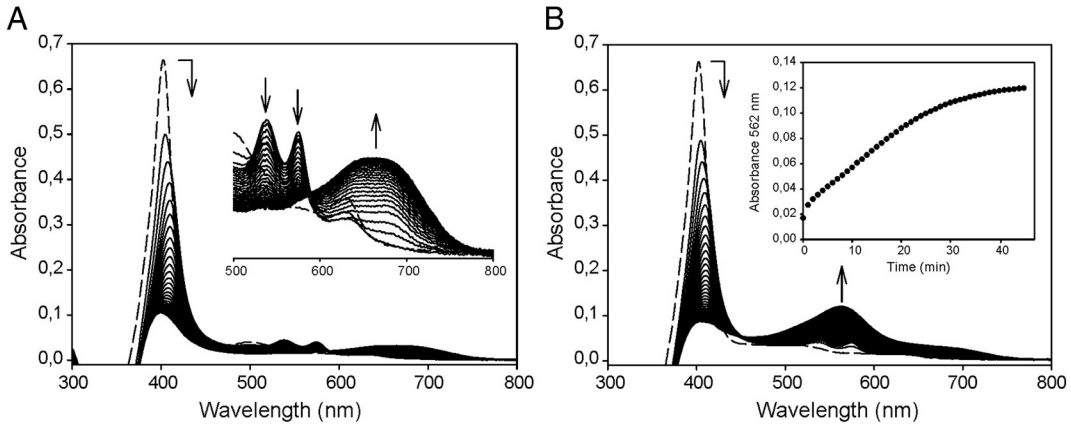

Fig. 6. Heme degradation carried out by LepHO. (A) Time dependent absorption spectra of LepHO before (dashed line) and after the addition of 0.5 μM LepFNR in the presence of 300 μM NADPH and 0.1 mg/ml catalase recorded at 1 min intervals (solid lines). Directions of spectral changes are indicated by arrows. The inset shows an enlargement of the region between 500 and 800 nm. (B) Same reaction as (A) but in presence of 250 μM ferrozine. The inset shows a time-dependent plot of the formation of the ferrozine-ferrous iron complex (562 nm) upon addition of LepFNR.

4.1. Phylogeny indicates that both LepHO and LepFNR arrived from a common ancestor

From our phylogenetic analysis we concluded that important divergences are observed among different HOs, in contrast to previous reports [17]. Bacterial HOs are clearly grouped according to their phylum, and cyanobacterial enzymes are closely related to the eukaryotic HOs, as has been reported previously [17]. The HO from *Leptospira* appears to have separated from their ancestors in an ancient event. Previously, we studied the phylogenetic relationships of LepFNR, also noting in this case that the reductase may have arrived from an ancient common ancestor [35]. These observations are coherent with the hypothesis that major evolutionary changes within prokaryotes may have occurred in a directional manner, being Spirochetes older species than Proteobacteria [35,67]. The fact that HO grouped differentially among pathogenic and non pathogenic leptospiras could be interpreted as evidence of some specialization of function. Probably, both proteins (LepHO and LepFNR) have improved their functional relationship and their role in pathogenesis in parallel. An evident similarity is observed when phylogenies of both LepHO and LepFNR were compared (data not shown). We have previously suggested that LepFNR may have arrived form a lateral gene transfer event [68]. However, the evidence provided here indicates that both LepFNR and LepHO have been present already in the proto-spirochete [69], from which all leptospiras come.

4.2. LepHO shows distinctive features not previously observed in other HOs

With the aim of studying the structural and functional properties of *L. interrogans* HO we first analyzed the interaction between the enzyme and its prosthetic group/substrate heme. Previously, by titrating hemin into 10 μM LepHO it has been estimated a K_d of 4 μM for the binding process [14]. We observed that reducing LepHO and hemin final concentrations during the titration experiments allows obtaining data that better adjust to a hyperbolic curve. We performed simulations of equilibrium conditions establishing that 0.13 μM apo-LepHO was the most adequate protein concentration to achieve saturating and spectrophotometrically measurable conditions. Using this setup, we estimated a K_d of 17 nM, significantly lower than all other reported K_d values [10,28,58]. Therefore, it is possible that if many of the dissociation constants previously published for HOs were recalculated using more appropriate conditions, lower values of them will be obtained. Recently, Koga et al. through development of a heme sensor using fluorescently labeled heme oxygenase estimated a K_d of ~1.5 nM for the rat HO-1-heme complex. In this case, the authors employed a protein concentration of 50 nM [70]. In a previous study using the absorbance titration of 3 μM wild-type HO-1, they had determined a K_d of 0.35 μM [71]. The difference of the K_d values between the fluorescence titration and the absorption titration was attributed to the changes in protein and hemin concentrations used in the two experiments. However, a possible effect introduced by fluorescent labeling cannot be disregarded.

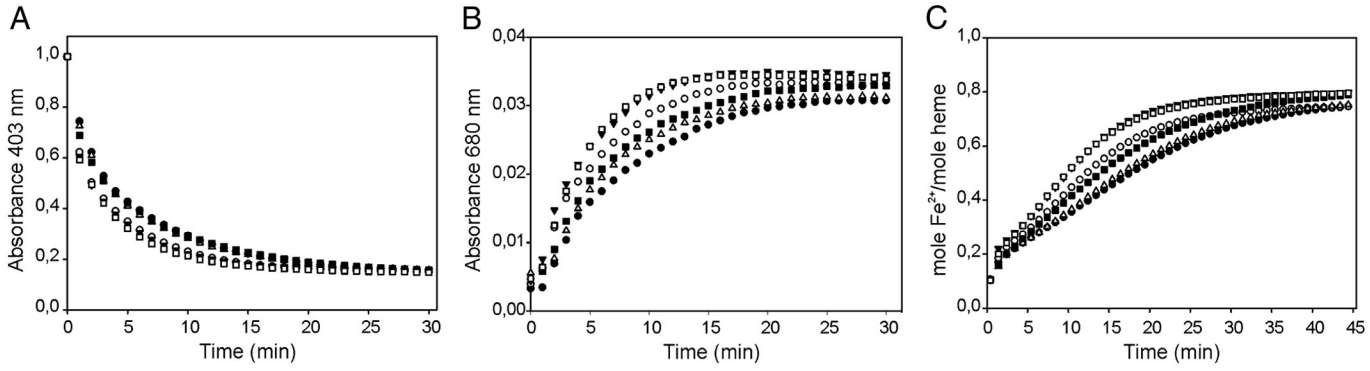

Fig. 7. Heme cleavage by LepHO with LepFNR and LFd2 as redox partners. Time dependent absorbance changes at 403 nm (A) and 680 nm (B) during heme degradation for reaction mixtures containing 6 μM LepHO, 300 μM NADPH, 0.1 mg/ml catalase and 0.5 μM LepFNR (●); 1 μM LepFNR (○); 1.5 μM LepFNR (▼); 0.5 μM LepFNR/0.5 μM LFd2 ( ); 0.5 μM LepFNR/1 μM LFd2 (■); or 1 μM LepFNR/0.5 μM LFd2 (□). The decrease of absorbance at 403 nm and the increase at 680 nm are diagnostic of the heme degradation and formation of biliverdin, respectively. Time-dependent plot of Fe^{2+}/heme molar ratio (C) during heme degradation for reaction mixtures as described above with the addition of 250 μM ferrozine. The amount of iron released was estimated using an extinction coefficient of 27.9 mM^{-1} cm^{-1} at 562 nm.

Several different sources of reducing equivalents have been proposed for the heme degradation catalyzed by HOs. The mammalian enzyme uses NADPH cytochrome P450 reductase as its sole source of electrons [26,72], whereas the plant, bacterial, algal and cyanobacterial HOs use reduced ferredoxin [9,28,73,74]. These ferredoxin-dependent HOs are thought to receive electrons from NADPH via ferredoxin reductase and ferredoxin. However, it was suggested that in addition to ferredoxin, an auxiliary electron donor is necessary to carry out the catalytic activity of HOs. A third redox partner, putidaredoxin, has been identified from in vitro studies of *Corynebacterium diphtherae* heme oxygenase [11]. More recently, Rivera et al. demonstrated that in *P. aeruginosa* a $NADP^+$-dependent ferredoxin reductase directly delivers the electrons to HO for heme cleavage, without the need of a mediating ferredoxin [30]. In this work we found that *L. interrogans* FNR is the redox partner of LepHO and the degradation of heme occurs efficiently in the absence of an auxiliary electron donor (ascorbate or trolox). Moreover, we observed that in vitro heme cleavage proceeds to iron and α-biliverdin as the final products of the LepFNR-LepHO reaction; release of Fe^{2+} does not require the addition of acid or a chelating agent such as desferroxiamine like was previously reported for the *P. aeruginosa* FPR-HO system [30] and other HOs [28,75]. Our findings indicate that LepFNR is capable to drive the heme degradation to completeness. Consequently, it may be proposed that the LepFNR/LepHO system may be sufficient to sustain the release of heme-iron in *Leptospira*.

5. Conclusions

In this work we have demonstrated that HO from *Leptospira interrogans* is able to bind and efficiently catalyze the cleavage of heme to free iron and biliverdin, using LepFNR as the sole electron source. Due to existing evidence of LepHO participation in pathogenesis, the understanding of this metabolic step may be relevant for the development of future therapeutic intervention.

At present, the only recognized protein substrates of FNRs are ferredoxin and flavodoxin. Our results, in addition to those already published for the *P. aeruginosa* HO, add a new substrate to this list, encouraging the search for new redox partners of FNR. Moreover, this finding may extend the range of possible metabolic pathways where the flavoenzyme could be involved.

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Appendix A. Supplementary Data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagen.2014.07.021.

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