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Heme environment in HmuY, the heme-binding protein of *Porphyromonas gingivalis*

Halina Wójtowicz [a], Jacek Wojaczyński [b], Mariusz Olczak [a], Jarosław Króliczewski [c], Lechosław Latos-Grażyński [b], Teresa Olczak [a,*]

[a] Laboratory of Biochemistry, Faculty of Biotechnology, University of Wrocław, Tamka 2, 50-137 Wrocław, Poland
[b] Department of Chemistry, University of Wrocław, 50-383 Wrocław, Poland
[c] Laboratory of Biophysics, Faculty of Biotechnology, University of Wrocław, 50-148 Wrocław, Poland

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ABSTRACT

Porphyromonas gingivalis, a Gram-negative anaerobic bacterium implicated in the development and progression of chronic periodontitis, acquires heme for growth by a novel mechanism composed of HmuY and HmuR proteins. The aim of this study was to characterize the nature of heme binding to HmuY. The protein was expressed, purified and detailed investigations using UV–vis absorption, CD, MCD, and ^{1}H NMR spectroscopy were carried out. Ferric heme bound to HmuY may be reduced by sodium dithionite and re-oxidized by potassium ferricyanide. Heme complexed to HmuY, with a midpoint potential of 136 mV, is in a low-spin Fe(III) hexa-coordinate environment. Analysis of heme binding to several single and double HmuY mutants with the methionine, histidine, cysteine, or tyrosine residues replaced by an alanine residue identified histidines 134 and 166 as potential heme ligands.

Introduction

Porphyromonas gingivalis is a black-pigmented Gram-negative anaerobic bacterium which has been implicated as a major etiological agent in the development and progression of chronic periodontitis. The uptake of heme is an important mechanism by which *P. gingivalis* obtains iron for its survival and its ability to establish an infection [1]. The bacterium has the ability to bind both monomeric iron protoporphyrin IX and its μ-oxo bis–heme complex and accumulate the latter as a black pigment [2,3]. Gram-negative bacteria utilize outer-membrane receptors to acquire heme from host hemoproteins directly or through a hemophore and then transport the captured heme into the cell [4]. We identified an outer-membrane heme utilization receptor (HmuR) which is involved in P. gingivalis' acquisition of both free heme and heme bound to hemoproteins [5]. The *hmuR* gene in the *P. gingivalis* A7436 strain is located in one operon with the *hmuY* gene and four uncharacterized genes [6]. An identical structure of this operon was shown in *P. gingivalis* W50, W83, and 381 strains [6,7]. HmuY is a membrane-associated putative lipoprotein identified in *P. gingivalis* 381, W50, W83, and ATCC 33277 strains [6–9], and in *Bacteroides fragilis* and *B. thetaiotaomicron* [6]. We reported that HmuY bound heme *in vitro* and that its expression was regulated by iron [6]. Recent reports demonstrated that HmuY is produced predominantly under low-heme conditions in bacteria forming biofilm [10,11].

Biochemical and structural studies of *P. gingivalis* HmuY in the presence of its ligand are required to establish the role of this protein in a novel bacterial heme uptake mechanism. As part of our ongoing studies to clarify the role of HmuY, we initiated an examination of the properties of heme bound to HmuY by identifying the oxidation and spin state of the heme iron and the ligation status of the iron.

Materials and methods

Construction and purification of HmuY. Point mutations were introduced using a QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene) and pHmuY11 plasmid as a template [6]. All amino acids with a potential ability to bind heme (3 histidines, 4 methionines, 1 cysteine, and 11 tyrosines) were substituted by an alanine, resulting in single HmuY mutant proteins. In the case of histidines, double mutants were also constructed. The expression, isolation, and purification of the HmuY wild-type and mutant proteins were carried out as described previously [6].

Determination of protein concentration and heme content. The protein concentration was determined on the basis of absorbance at 280 nm using an experimental coefficient determined in this study (ε_{280} = 36.86 mM^{-1} cm^{-1}) as described by others [12]. The modified Bradford method (Roti-Nanoquant, Roth) [13] was used

* Corresponding author. Fax: +48 71 3752 608.
E-mail address: Teresa.Olczak@biotech.uni.wroc.pl (T. Olczak).

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to determine protein concentration for experiments utilizing mutant HmuY and holo-HmuY samples. A pyridine hemochrome assay [14] was used to assess the content of heme associated with HmuY. Heme (ICN Biomedicals) solutions were freshly prepared as described previously [2]. To achieve quantitative loading of HmuY with heme, the protein was incubated with heme at a 1:2 molar ratio. Excess heme was removed by gel filtration (PD-10; Amersham Pharmacia).

Electrochemical redox titrations. The midpoint potential of the HmuY–heme complex was measured by spectroelectrochemistry as described earlier [15,16]. Protein samples were dialyzed to 1 volume of 50 mM MOPS buffer, pH 7.0, containing 50 mM KCl, and the appropriate mediators were used at concentrations of 100 μM each. Measurements were carried out anaerobically at 25 °C with a micro ORP combination electrode with an Ag/AgCl electrode as a reference (Microelectrodes, Inc.). The potential was adjusted by the addition of potassium ferricyanide or sodium dithionite. Spectral changes recorded with a DU800 spectrophotometer (Beckman) were analyzed by fitting the absorbance values at 525 nm to Nernst curves. The midpoint potential of the heme was calculated using a global fit procedure [16].

UV–vis absorption spectroscopy. UV–vis absorption spectra of HmuY were recorded using an Agilent 8453E UV–vis spectrophotometer (Agilent Technologies). Heme titration experiments were performed in 20 mM phosphate buffer, pH 7.6, containing 20 mM NaCl. To analyze the redox properties of the iron present in the heme molecule bound to HmuY, sodium dithionite and potassium ferricyanide were used as the reductant and the oxidant, respectively [17].

Circular dichroism (CD) and magnetic circular dichroism (MCD) spectroscopy. CD spectroscopy was carried out using a Jasco J-810 or J-715 spectropolarimeter and MCD spectroscopy using a Jasco J-715 spectropolarimeter equipped with an electromagnet generating a magnetic field of 1.46 T. Samples were examined at 25 °C in 20 mM phosphate buffer, pH 7.6, containing 20 mM NaCl. To analyze the redox properties of the iron present in the heme molecule bound to HmuY, sodium dithionite was used as the reductant [17].

NMR spectroscopy. ^{1}H NMR measurements were performed using a Bruker Avance 600 spectrometer operating at a proton Larmor frequency of 600.13 MHz. The data were collected at 298–328 K with the chemical shift referenced to a residual water signal. The strong H_2O/HDO resonance was suppressed by pre-saturation. 2D COSY and NOESY spectra [18,19] were recorded with pre-saturation of the solvent signal, using a full spectral width (50 ppm) with 256–512 t_1 blocks with 4096 t_2 points and 320–400 scans per block. The mixing time applied in the NOESY spectra was in the range of 50–100 ms. HmuY samples (0.2–1.6 mM) were prepared by dissolving lyophilized holo-proteins in D_2O.

Results and discussion

Binding of heme to HmuY analyzed by UV–vis absorption spectroscopy

A novel mechanism of heme uptake in *P. gingivalis* suggests heme transfer from hemoglobin to HmuY and subsequently from HmuY to the outer-membrane receptor HmuR [1,6, and unpublished data]. Since HmuY may switch between the apo- and holo-forms as part of its function, the biophysical properties of both states are of biological relevance and may explain how heme transport occurs. Previously we showed that the addition of oxidized heme to HmuY resulted in a change in the visible spectrum compared with that of ferric heme alone [6]. Here we demonstrated that reduction with sodium dithionite changed both the intensity and position of the Soret peak, which shifted from 411 to 425 nm, and produced increased and well-resolved α and β bands at 559 and 529 nm, respectively (Fig. 1A). The heme bound to HmuY was further re-oxidized with potassium ferricyanide, resulting in a Soret band shift back to 411 nm and a decrease of the α band at 559 nm (Fig. 1A). The low-spin-state spectra of the HmuY–heme complex (Fig. 1A) closely resemble those of *b*-type cytochromes, which possess heme non-covalently bound to apoprotein and are characterized by two histidine axial ligands [20,21]. The absence of peaks at 620 and 695 nm (high-spin and methionine axial ligand indicators, respectively) in the spectrum of HmuY is also typical for *b*-type cytochromes. The midpoint potential (E_{m7}) of the HmuY–heme complex (136 ± 1.23 mV) (Supplementary Fig. 1) is similar to those observed for bis-histidine *b*-type hemoproteins [21].

CD and MCD analyses of HmuY–heme complex

The CD and MCD spectra of the protein–heme complex provide useful information on the iron-heme spin state and coordination. The analysis is based on the comparison of the novel complex's spectroscopic signatures with the data of the already characterized systems. The CD spectrum of the HmuY–heme complex analyzed in the Soret region was characterized by a negative Cotton effect, with a broad minimum at ~400 nm (Fig. 2A). Reduction of the HmuY–heme complex by sodium dithionite resulted in a shift towards higher wavelengths (Fig. 2B). The inverted nature of the Soret spectrum of the wild-type HmuY indicates a different heme environment from that of hemoglobin, myoglobin, or peroxidase, which exhibit a positive Cotton effect [22]. Conversely, the CD spectrum obtained for the HmuY–heme complex is highly similar to that of cytochromes *b* [20,22–27], which strongly confirms the results obtained from UV–vis absorption spectroscopy. The MCD characteristics of the heme bound to HmuY were *A* terms in the Soret and Q band regions with zero-crossing points at the absorption maxima at 411 and 559 nm, respectively (Fig. 3A). In the Soret region, the reduced HmuY–heme complex exhibited a very weak inverse derivative-shaped *A* term (Fig. 3B). In the visible region, the spectra of the reduced HmuY–heme complex displayed an intense *A* term associated with the α band (zero-crossing point at 556 nm) and a weak *A* term associated with the β band (zero-crossing point at 526 nm) (Fig. 3B). Thus our interpretation of the data presented here is that the heme contains a hexa-coordinate low-spin ferric iron ($S = 1/2$) with bis-histidine ligation [20,23].

^{1}H NMR characteristics

^{1}H NMR spectroscopy applied to paramagnetic hemoproteins provides valuable information about their structure and magnetic properties. The heme hyperfine shift pattern observed for the pyrrole-H, methyl, vinyl, and *meso*-H resonances reflects the oxidation/spin/ligation state of iron [28,29]. In the spectrum obtained at 328 K, the temperature resulting in the optimal resolution of peaks, all signals were located in the region from 25 to −8 ppm (27.5-(−10) ppm at 298 K) (Fig. 4A). The characteristic four downfield-shifted peaks located at 24.7, 24.4, 21.3, and 17.0 ppm exhibited equal intensities and could be attributed to four heme methyl groups. They are indicative for the low-spin Fe(III) ($S = 1/2$) coordinated with two strong-field axial ligands. Their average chemical shift (21.8 ppm at 328 K, 23.9 ppm at 298 K) is comparable to values observed for different ferricytochromes [30]. The β-vinyl signals also exhibit a localization which is typical of low-spin Fe(III) protoporphyrin IX complexes. Interestingly, three sets of resonances (2-β, 4-β_A, 4-β_B) (Fig. 4A), instead of the expected two, are clearly visible in the upfield part of the spectrum and the intensity of each set is different (−1.91 and 2.10, −2.97 and −3.22, −4.27