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# Hemoglobin Binding Activity and Hemoglobin-Binding Protein of *Prevotella nigrescens*

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**Abstract**
*Prevotella nigrescens*, lacking siderophores was found to bind to the hemoproteins. The binding was observed also in the envelope which was prepared by sonication of the cell. The binding occurred in the pH-dependent manner; the binding was observed below neutral pHs of the incubation mixtures but only slightly observed in the neutral and alkaline pHs. Furthermore, hemoglobin bound to the envelope was dissociated at high pHs buffers. Maximum amounts of hemoglobin bound to 1 mg envelope was 51.2μg. K*d* for the reaction at pH 5.0 was $2.1 \times 10^{-10}$M (210 pM). From the dot blot assay, hemoglobin could bind to a protein solubilized from the envelope by a detergent, referred to as hemoglobin-binding protein (HbBP), then it was purified by the sequential procedures of ion exchange chromatography, affinity chromatography and isoelectric focusing. Molecular weight and isoelectric point of the HbBP were 46 kDa and 6.1, respectively.

*Key words:* iron acquisition, periodontal pathogen, *Prevotella nigrescens*, hemoglobin, hemoproteins, hemoglobin-binding protein, purification, anaerobe

## Introduction

*Prevotella nigrescens* is a gram-negative obligate anaerobic oral indigenous bacterial species. It forms characteristic jet-black colonies on the blood agar plates, as well as *Porphyromonas gingivalis* and *Prevotella intermedia*. These bacteria have been implicated in the pathogenesis of periodontitis, based on their ecological properties in the oral cavity and etiological characteristics including production of proteolytic enzymes [1-6] and endotoxin [7-10].

Even though pathogenic bacteria absolutely require iron, it is chelated by ferric binding proteins such as transferrin and lactoferrin or globulins. Therefore, concentration of free iron in the mammalian body fluids is extremely low, less than $10^{-18}$ M, which is far from the concentration to allow the bacterial growth [11-13]. Many pathogenic microorganisms elaborate siderophores which can chelate boundary iron with tremendously strong affinity to iron [14]. However, since the above stated periodontal pathogens are known to lack the siderophores [13, 14], they must possess other mechanism of iron acquisition. We have reported the binding activity to hemoproteins [15, 16] and isolation of hemoglobin binding protein (HbBP) from the envelope of *P. gingivalis* [17] which may function in the uptake process iron source from hemoglobin. In *P. nigrescens*, we also observed binding activity to hemoproteins. Then, we discuss the binding properties and purification of HbBP.

## Materials and Methods

### Bacterial Strain and Cultivation Methods

*P. nigrescens* ATCC33563 was maintained on blood agar plates supplemented with hemin and menadione at 37 °C in an anaerobic glove box filled with a mixture of gasses ($N_2 + H_2 + CO_2$, 85 : 10 : 5). The bacteria were inoculated into a medium consisting of 17 g of Trypticase peptone, 3 g of yeast extract, 2.5 g of glucose, 2.5 g of $K_2HPO_4$, 5 g of NaCl, 5 mg of hemin, and 0.5 mg of menadione per liter and cultured anaerobically at 37 °C for 3 days.

### Preparation of The Envelope and Solublization

The cells were harvested by centrifugation at 12 000 × g and washed with saline and suspended in 50 mM Tris-HCl buffer, pH 8.2. The cells were disrupted by ultrasonic treatment at 150 W for 20 min. The sonicate was centrifuged at 7 000 × g for 10 min and the precipitate containing unbroken cells and cell debris was discarded, on the other hand the supernatant represents the envelope was centrifuged at 120 000 × g for 1 h to separate the envelope from cell extract. Then the envelope was washed with 50 mM Tris-HCl buffer, pH 8.2 by centrifugation at 120 000 × g and resuspended in the same buffer. To a suspension of the envelope 3-[3-cholamidopropyl]-dimethylammoniol]-propanesulfonate (CHAPS) was added to 0.5 % and stirred for 5 h. After incubation, the mixture was centrifuged at 120 000 × g for 1h. The supernatant solution corresponding to the solubilized fraction of the envelope was kept at -40 °C until use. The insoluble material was rinsed with 50 mM Tris-HCl buffer, pH 8.2 by centrifugation at 120 000 × g for 1 h and was referred to as outer membrane.

### Dot Blot Assay of Hemoglobin Binding

Horse radish peroxidase (Sigma Chemical Co., St. Louis, Mo) was oxidized with sodium periodate to form aldehyde groups within the enzyme molecules, and hemoglobin was coupled to this oxidized enzyme [18, 19]. Binding of hemoglobin to HbBP of the envelope component was detected by using peroxidase-labeled hemoglobin and the method of Frangipaine et al. [20], including the application to nitrocellulose membranes, blocking with skim milk, and development with 4-chloro-1-naphthol-hydrogen peroxide.

### Examinations of Binding And Dissociation of Hemoglobin to and from the Envelope

The amounts of hemoglobin bound to the envelope in the different pH buffers were determined by the essentially the same as our previous papers [15, 17]. The envelope (4 mg in wet weight) was mixed with 800 µl of hemoglobin solution (300 µg/ml) in water, and 200 µl of 0.5 M buffer as described below; acetate buffer (pH 4.5 to 6.0), Tris-maleate buffer (pH 6.5 to 7.5), Tris-HCl buffer (pH 8.0 to 9.0), and carbonate-bicarbonate buffer (pH 9.5 to 10.0). The mixtures were incubated at 37 °C for 15 min and centrifuged at 120 000 × g. Unbound hemoglobin in the supernatant was measured by absorbance at 410 nm and the amount of bound hemoglobin was determined by subtraction. Binding of hemoglobin was tested also using the intact cell and the outer membrane.

Dissociation of hemoglobin from the complex of the envelope and hemoglobin formed in 50 mM acetate buffer, pH 4.5 was also examined in pH range from 4.5 to 10. The degree of dissociation of hemoglobin was expressed as the amount of dissociated hemoglobin in each buffer versus completely released hemoglobin obtained by solubilization of the complex by CHAPS [15, 17].

### Determination of Binding of Hemoproteins to the Envelope

Photometrical method was employed to assay the amount of hemoproteins including hemoglobin, myoglobin, cytochrome *c*, and catalase bound to the envelope as described earlier [15]. Briefly, 5 mg of the envelope was incubated with 270 µg of human hemoglobin at 37 °C for 15 min and centrifuged. Unbound hemoglobin in the supernatant was measured by absorbance at 410 nm and the amounts of hemoglobin released from the formed envelope-hemoglobin complexes were compared using the same buffers described above.

### Purification of HbBP

All steps of purification were conducted at 4 °C, if not otherwise stated.

Step 1: *Ion exchange chromatography*. The solubilized material of the envelope was applied to a column of Q-Sepharose (1.5 by 30 cm), equilibrated with 50 mM Tris-HCl buffer (pH 8.2) and the column was washed with the same buffer thoroughly until the eluates of the $A_{280}$ was reached less than 0.05. Then the column was eluted with a linear concentration gradient of NaCl from 0 to 1.0 M, which was generated by mixing 200 ml of 50 mM Tris-HCl buffer (pH 8.2) containing 1.0 M NaCl into an equal volume of 50 mM Tris-HCl buffer (pH 8.2). The flow rate was 20 ml/h, and 5 ml fractions were collected. The active fractions confirmed by dot blot assay were combined and dialyzed against 50 mM acetate buffer (pH 4.5) for 20 h. The insoluble material generated in the dialysis bag was removed by centrifugation at 12 000 × g for 20 min.

Step 2: *Affinity chromatography*. The dialyzed material was applied to a column (1 by 13 cm) of hemoglobin-conjugated agarose (Sigma) equilibrated with 50 mM acetate buffer (pH 4.5). The column was washed with the same buffer until the $A_{280}$ of eluates was less than

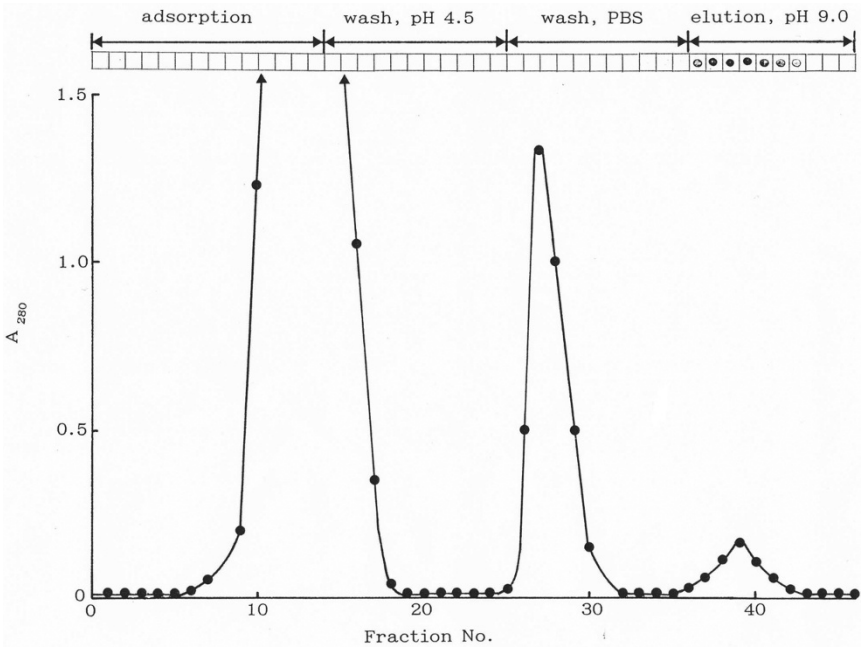


*Fig. 1.* Affinity chromatography of HbBP. The hemoglobin binding activity determined by dot blot assay is presented at the top of figure.

0.05 and then the column was washed with 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl (PBS). Finally the column was eluted with 50 mM Tris-HCl buffer, pH 9.0. Hemoglobin binding activity was found only in the fractions eluted with the pH 9.0 buffer (Fig. 1). The active fractions were combined and dialyzed against 1 % glycine solution.

Step 3: *Isoelectric foucusing*. The dialyzed material was subjected to isoelectric focusing electrophoresis. Electrophoresis was carried out using 1 % (v/v) ampholine (pH 3-10) under constant voltage (300 V) with cooling the column by tap water. After the electrophoresis, column contents were collected into 2 ml fractions. HbBP activities of each fraction were determined by dot blot assay and pH values were measured. The active fractions were dialyzed against 50 mM acetate buffer pH 4.5 and stored at -30 °C as the purified HbBP.

### SDS-Polyacrylamide Gel Electrophorrsis (SDS-Page)

SDS-PAGE was carried out using 12.5 % polyacrylamide. Molecular mass markers were phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa).

## Results

### Binding of Hemoglobin and other Hemoproteins to the Cellular Components

Effects of different pH values on the binding of hemoglobin to the envelope was noteworthy. Hemoglobin in the pH 4.5 buffer solution was found to bind completely to the envelope. The binding rates reduced as a function of lowering of the pH values to 8.0. Only slight binding occurred at pHs 8.5 to 10 (Fig. 3). When the established hemoglobin-envelope complex formed in the pH 4.5 solution was immersed in the pH 9.0 solution, hemoglobin released entirely from the envelope. However, dissociation was not vigorous in neutral and acidic solutions. These findings demonstrate that effects of pH on binding and dissociation among hemoglobin and the envelope is rightly reciprocal; HbBP in the envelope prefers acidic conditions to alkaline conditions in the hemoglobin binding reaction. Additionally, hemoglobin bound to the envelope easily dissociate in the alkaline solutions (Fig. 2).

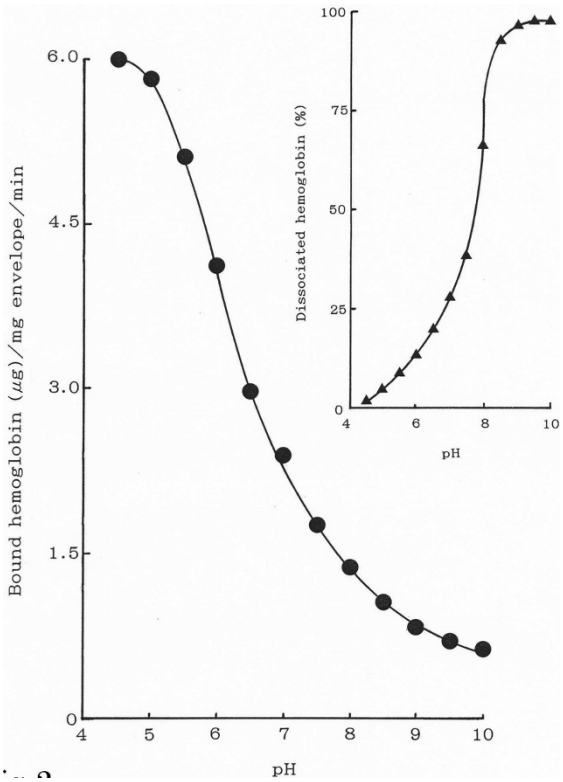


*Fig. 2.* Reversible binding of hemoglobin to the envelope.

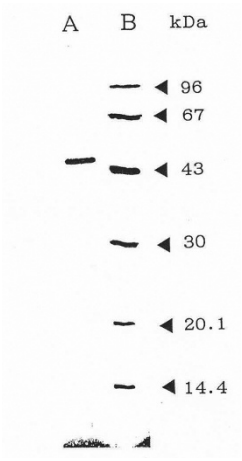


*Fig. 3.* SDS-PAGE of purified HbBP. A: Purified HbBP, B: Marker proteins.

Binding of five kinds of hemoproteins including hemoglobin, myoglobin, cytochrome *c*, catalase, and holo-transferrin to the envelope was tested in 50 mM acetate buffer, pH 4.5. As shown in Table 1, high amounts of hemoglobin and myoglobin were found to bind to the envelope. Considering myoglobin is a quarter moiety of hemoglobin molecule, the close degree of the binding seems to be reasonable. However, binding of cytochrome *c* and catalase was obviously weaker than that of hemoglobin and myoglobin. No significant binding was confirmed in holo-transferrin.

Comparative evaluation of binding of hemoglobin to the intact cell, envelope, and outer membrane was tested. The ratio of the activity of these components was 100 : 70 : 5 : 1.9.

*Table 1.* Binding of the Envelope to Hemoproteins.

| Hemoproteins | Initial amounts (μg) | Bound amounts (μg) |
|---|---|---|
| Hemoglobin | 270 | 266 |
| Myoglobin | 270 | 240 |
| Cytochrome *c* | 270 | 121 |
| Catalase | 270 | 99 |
| holo-Transferrin | 270 | 14 |

(Reactions were conducted in 50 mM acetate buffer, pH 4.5, using 5 mg envelope)

### Dissociastion Constant of Hemoglobin to the Envelope

From the plots of increasing amounts of hemoglobin and the amounts of hemoglobin bound to the envelope (Scatchard plot), hemoglobin binding to the envelope determined an apparent dissociation constant (K*d*) of 210 pM (data are not shown).

### Purity of HbBP, Molecular Weight, and Isoelectric Point

Isoelectric point was 6.1 judged from the isoelectric foucusing electrophoresis. The finally purified HbBP showed a single stained band in the SDS-PAGE, indicating this sample was purified to homogeneity. The molecular mass was estimated as 46 kDa as illustrate in (Fig. 3).

### Binding of HbBP to Hemoproteins

Dot-blot determination demonstrated purified HbBP bound actively to hemoglobin and myoglobin, moderately to cytochrome *c*, and weakly to catalase. However, binding to holo-transferrin was found to be negative.

### Thermostability and Resistance to Various Reagents

Heating of the envelope at 65 °C for 20 min resulted in the loss of hemoglobin-binding activity of 12 %. Similarly, the titer of the purified HbBP demonstrated one eighth activity after the same heating.

When the envelope and the purified HbBP were treated by trypsin (1 mg/ml) and chymotrypsin (1 mg/ml) at 37 °C for 30 min, separately, no significant decrease of hemoglobin binding activity was seen.

Neither inhibition nor activation of the hemoglobin binding activity of the envelope was observed by the incubation for 30 min by the following reagents; $Ca^{2+}$(1 mM), $Mg^{2+}$ (1 mM), $Mn^{2+}$ (1 mM), $Fe^{2+}$ (1 mM), EDTA (20mM), EGTA (20 mM), 1,10-phenanthrolin (50 mM), mercaptoethanol (5 mM), Leupeptin (2 mM), Antipain (10 mM), L-*trans*-Epoxy-succinylleucyl-amido-(4-guanidino)butane (E64) (2 mM), 4-(2-Aminoethyl)-benzenesulfonylfluoride(Pefabloc SC) (2 mM).

## Discussion

Usually, hemin is added to the ordinary medium as an iron source in the cultivation of the black pigmented periodontal pathogens. Since, hemin can be replaced by hemoglobin, it also is a possible source of iron *in vivo* for the species lacking siderophores as well as hemin. But it may be practically impossible to expect that the body fluids contain sufficient amounts of hemin to support the growth of these bacteria. On the contrary, in the diseased sites of periodontitis, red blood cells may be adequately supplied, which contributes to provide hemoglobin.

We confirmed binding activity of the envelope to hemoglobin. Its binding activity was remarkably affected by the differences in pH values of the incubation mixtures; binding was observed in the lower pHs buffer (pH 4.5 to 6.0) but only poor binding was found in neutral pH buffer, and no significant binding in the alkaline pHs buffer. Moreover, dissociation of hemoglobin from the established complex of the envelope and hemoglobin in alkaline condition was observed. These properties are quite similar to the findings in the case of *P. gingivalis* [17].

The properties of HbBP of *P. nigrescens* are generally similar to those of *P. gingivalis*, however, a substantial difference is noticed in the molecular masses of HbBP; that of *P. gingivalis* was 19 kDa [17], but it was 46 kDa in *P. nigrescens*. The chemical difference was seen in pIs between *P. nigrescens* and *P. gingivalis*. We estimated pI as 6.1 in this report, but in *P. gingivalis* it was 4.3 [17]. These observations indicate that the molecules of HbBPs of the two species are different from each other, but possess the similar properties in the mode of hemoglobin binding.

The fact that intact cell was found to actively bind to hemoglobin may be functionally useful to catch hemoglobin from the bacterial environment. However, the binding activity of the outer membrane was quite low (Fig. 1). Guan et al. reported that hemoglobin binding activity was confirmed in the outer membrane in *P. intermedia*, and a protein responsible for the binding to hemoglobin was isolated from the outer membrane, its molecular mass was 60 kDa [21], however HbBP of *P. nigrescens* was 46 kDa. These discrepancies may be due to the difference of bacterial species, even if both species are rather close each other.

According a report of HbBP of *P. nigrescens* [22], it was also active in low pH buffers than at higher pH. Its activity was resistant to heating at 60 °C for 10 min, but obvious decrease was observed heating at 80 °C. Treatment of the intact cells by trypsin resulted in about 35 % loss of the binding activity. Furthermore, in the extracted materials of the cells by *n*-octyl-β-D-thioglucoside, three proteins possessing molecular masses of 41 kDa, 56 kDa, and 59 kDa reacted to hemoglobin were detected. The differences of the properties from our results, particularly in the molecular masses, may be due to the different protein source, detergent extracts of the intact cells and the envelope.

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