Isolation and Characterization of a Putative Hemin-binding Protein from *Prevotella intermedia*

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I. Introduction

Iron is essential for the growth, reproduction, and survival of all prokaryotic and eukaryotic species[1-3]. Iron has also been shown to be essential for the initiation and progression of several infectious diseases[1]. In order to survive therefore, prokaryotes and eukaryotes have devised unique mechanisms to compete for, and sequester the small amounts of iron which are available to them.

Eukaryotic cells compete for iron by synthesizing large number of high-affinity iron-binding proteins (i.e., transferrin, lactoferrin), hemoglobin-binding protein (haptoglobin), heme-binding proteins (hemopexin, albumin), and iron-storage proteins (ferritin, hemosiderin) which are capable of competing for, and storing iron[1,4-8]. Therefore, in order for host-associated bacteria to survive, they must be capable of competing with the host's large number of high-affinity iron-binding proteins. Prokaryotic cells compete with eukaryotes for this iron by employing two mechanisms: synthesizing their own high-affinity binding and transport proteins, siderophores, and outer membrane associated iron-binding protein.

The siderophores are low molecular weight proteins, which bind iron by chelating it from the host, and transferring it to the specific outer membrane receptors which then transport it directly into the cell[9]. Several Gram-negative bacteria contain, in addition to, or in place of siderophores, iron (hemin) binding proteins in their outer membrane[10-21]. These outer membrane-associated iron-binding proteins are capable of binding iron directly, and transporting it across the outer membrane into the cell. For the most part, these outer membrane expressed iron-binding proteins are expressed during iron stress, and are formed in response to very low levels of iron in the environment.

While there are several studies which have dealt with the hemin-binding and hemin-regulated proteins in the oral pathogen, *Porphyromonas gingivalis*[20,22-25], there is no such studies which has studied hemin-binding in *Prevotella intermedia*. In the study presented here, we have identified, purified, and characterized a putative hemin-binding protein in *Prevotella intermedia*.

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II. Materials and Methods

1. Bacterial Strains and Growth Conditions

Prevotella intermedia (*P. intermedia*) ATCC 25611 was used in this study. Cells were grown anaerobically on the surface of enriched Trypticase soy agar, or in 2.1% (w/v) *Mycoplasma* broth base (BBL, Becton Dickinson, Cockeysville, MD) supplemented with 1㎍/ml menadione and 5㎍/ml hemin[23]. Plate grown cultures were routinely incubated for 4 days and used as the inoculum for liquid growth. Liquid grown cells were incubated for approximately 24h, equivalent to late exponential growth phase. For hemin restriction (i.e., hemin starvation), late exponential or early stationary phase cultures were grown with excess hemin (i.e., 7.7㎛M hemin), and serially passaged at least 5 times as a 10% inoculum into hemin-free medium. All glassware was washed in chromic acid and rinsed in deionized water to remove contaminating iron and hemin. The cultures were grown in a Coy anaerobic chamber (Coy Laboratory Product Inc.) in a N_2-H_2-CO_2 (85%-5%-10%) atmosphere at 37°C. Growth was monitored over a 60h period in duplicate by measuring the absorbance at 660 nm. Culture purity was assessed by Gram staining and plating to solid medium.

2. Cell Envelope Preparation

P. *intermedia* ATCC 25611 was grown into late exponential-early stationary phase. The cells were harvested by centrifugation at 12,000 X g, for 20 min at 4°C, washed 3 times in cold phosphate-buffered saline (PBS, pH 7.2), and resuspended in PBS containing a protease inhibitor cocktail consisting of 2mM each of phenylmethylsulfonyl fluoride (PMSF), Na-P-tosyl-L-lysine chloromethyl ketone (TLCK), and benzamidine. Cell envelopes were prepared by sonication of whole cells in PBS by four disruption cycles. The cell envelopes were recovered after low-speed (10,000 X g, 30 min) and high-speed (200,000 X g, 2h) centrifugation. The envelopes were suspended in PBS and stored at 20°C until used.

3. Protein Determination

Protein concentration was determined using the bicinchoninic acid (BCA) assay of Pierce (Pierce, Rockford, IL) modified by Kennel and Holt[26]. The assay was modified to a microassay for protein determination in flat-bottomed microtiter plates. Briefly, 20 ㎕ cells or various cell fractions were serially diluted in the microtiter wells with distilled water. 200 ㎕ of protein assay reagent was added to each well, the plates were incubated at room temperature for 30 min, and the resulting color read on a plate leader at 570 nm. Bovine serum albumin was used as the protein standard.

4. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) procedure of Laemmli[27] was employed for the determination of protein distribution. 12% acrylamide separating gels were routinely used. All gels were run with a 4% acrylamide stacking gel in a vertical slab gel apparatus (Hoefer Scientific, San Francisco, CA). Gels were run at 30 mA until the dye front was at the leading edge of the gel. 70㎍ of protein was applied per lane unless otherwise indicated. Broad molecular weight standards (Bio-Rad, Richmond, CA) were run with each gel. Proteins were visualized by Coomassie brilliant Blue-R-250 stain (CBB). The molecular weight distribution of envelope proteins was determined by lin-

ear regression analysis. Electrophoretic mobility was analyzed in the presence and absence of reducing agent, 2-mercaptoethanol.

5. Purification of the Putative Hemin-binding Protein

Hemin-binding protein from *P. intermedia* ATCC 25611 was purified from the membrane fraction of hemin-starved passage 5 cells by 1D SDS-PAGE through a 12% gel employing a preparative comb with one reference well. The elution protocol of Hage and Burgess[28] was used. Briefly, the membrane fraction was electrophoresed as described above, and the end sections on either side of the gel were cut, stained with CBB, and used as a template to excise a horizontal strip containing the hemin-binding protein. The excised strip was mashed, and then the protein extracted with an elution buffer consisting of: 0.1% SDS, 0.05M Tris-HCl, 0.1mM EDTA, 5mM DTT, 0.2M NaCl, for 1h at room temperature. A second elution was then performed identical to the first, and the fractions were pooled and concentrated in a Centricon-10 microconcentrator (10,000 molecular weight cutoff; Amicon Div., Beverly, MA). Purity of the isolated protein confirmed by 1D SDS-PAGE.

6. N'-terminal Amino Acid Sequencing

For the N'-terminal sequence analysis, the purified protein was precipitated with acetone according to Hager and Burgess[28]. Briefly, five volumes of cold acetone (-20°C) was added to the microconcentrated protein and allowed to precipitate for 30 min in a dry ice-ethanol bath. The precipitated protein was separated by centrifugation, the supernatant carefully decanted, and the tube inverted to drain. The dried protein was dissolved in 1x sample buffer, and then heated at 100°C, 5 min, and electrophoresed through a 12% acrylamide gel. After electrophoresis, the protein was transferred onto a polyvinylidine difluoride membrane (ProBlot, Applied Biosystems, Foster City, CA), at 100 mA, 4h. After transfer, the ProBlot membrane was removed from the transblotting sandwich and rinsed with deionized water. Protein bands on the ProBlot membrane was visualized by Amido black staining. Before staining, the membrane was saturated with 100% methanol for a several seconds. Destaining was carried out with deionized water. The protein band was excised from the dried membrane, and its N'-terminal sequence was determined with an Applied Biosystems (Foster City, CA) Model 477A gas-liquid phase sequanator coupled to an on-line high-performance liquid chromatography model 120A analyzer.

III. Results

1. Effect of Hemin Restriction on Growth

The effects of hemin starvation on the growth of *P. intermedia* ATCC 25611 is seen in Figure 1. As seen in Figure 1, sequential passages in hemin-free medium resulted in a significant increase in the mean generation time. In the presence of normal hemin (i.e., 7.7 μM hemin), *P. intermedia* ATCC 25611 grew with a mean generation time of 6.2h^{-1}. At passage 1, the generation time was approximately the same as the original culture, indicating that the cells were employing their intracellular hemin stores for hemin. However, from passage 2 through 6, there was a dramatic increase in mean generation time, such that at passage 5 the cells were dividing at a rate of only 15.4h^{-1}. At passage 6, the cells were growing very poorly, and the recovery of dry cell mass was very low compared to cells grown in hemin normal conditions or at passage 1.

2. SDS-PAGE Analysis of *P. intermedia* ATCC 25611 Membranes

The effect of hemin restriction by hemin starvation (i.e., passage 5) on the expression of celll envelope proteins compared to hemin normal grown *P. intermedia* ATCC 25611 is seen in Figure 2. As seen in Figure 2, *P. intermedia* synthesizes a large number of proteins, including LPS-associated proteins, when grown in hemin-normal (7.7 μM) medium. The distribution and number of protein bands in all of the treatments appeared very similar. Upon hemin restriction, as a result of passage in the absence of hemin, there appeared to be the hemin regulation of at least 1 protein. Under hemin restriction the 50 kDa protein (heated) was significantly increased in CBB staining density compared to that seen in the cell envelopes of hemin normal grown cells (compare Figure 2, lanes 5 and 3).

3. Purification of the Putative Hemin-binding Protein

The 50 kDa *P. intermedia* ATCC 25611 membrane protein was purified from the cell envelopes (Figure 3). The 50 kDa protein was isolated from the SDS-PAGE gel of the heated passage 5 membrane fraction (Figure 3, lane 3). The resulting 50 kDa protein was isolated from the gel as a single protein band, with no contaminating proteins even when the gels were overloaded with large amounts of purified protein. Electrophoretic mobility of the isolated protein was same in both the presence and absence of reducing agent, 2-mercaptoethanol, indicating the absence of disulfide bonds in the protein (Figure 4).

4. N'-terminal Sequence Analysis

N'-terminal amino acid sequence analysis of 50 kDa protein is seen in Table 1. A search of Gen-

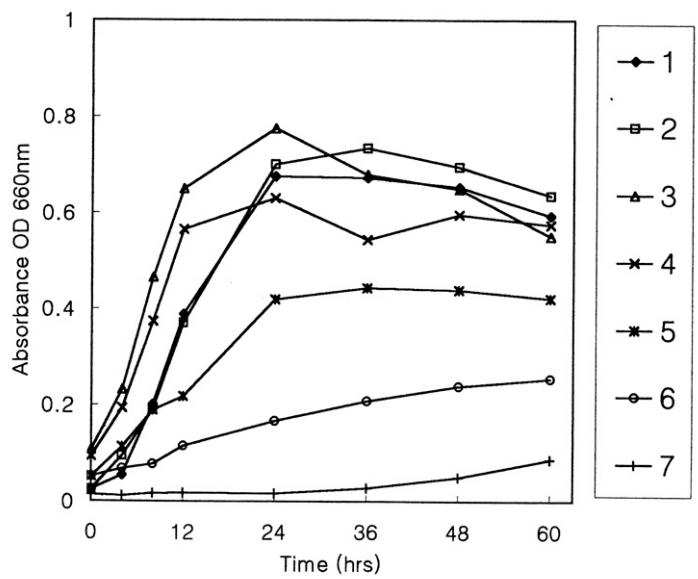

Figure 1. Effect of hemin restriction on the growth of *P. intermedia*. Cells were progressively passaged from hemin excess culture to hemin-free medium for 6 passages; 1, hemin-excess (7.7 μM); 2, passage 1; 3, passage 2; 4, passage 3; 5, passage 4; 6, passage 5; 7, passage 6.

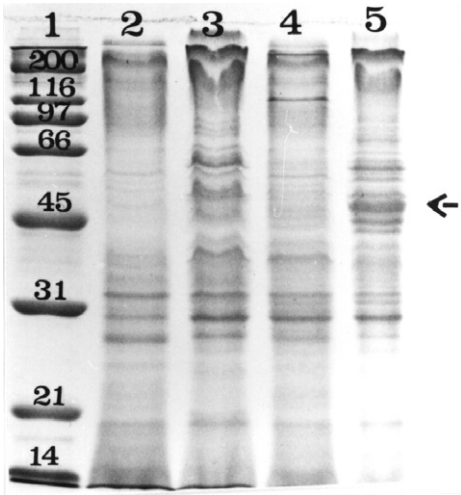

Figure 2. SDS-PAGE of cell envelopes from *P. intermedia*. Lane 1, broad M.W. standards; Lanes 2 and 4, unheated samples; Lanes 3 and 5, samples heated at 100°C for 5 min; Lanes 2 and 3, cells grown in 7.7 μM hemin; Lanes 4 and 5, cells grown without hemin (passage 5).

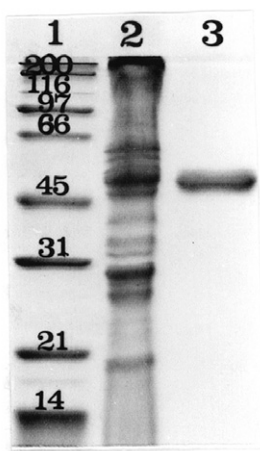

Figure 3. Purification of a putative hemin-binding protein. Lane 1, broad M.W. standards; Lane 2, cell envelope fraction (passage 5); Lane 3, isolation of 50 kDa protein.

Bank for proteins with similar amino acid sequence to 50 kDa protein revealed that the sequence is identical with Enolase from *Streptococcus intermedius*.

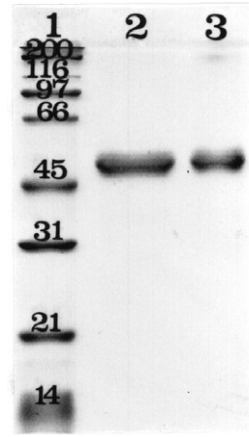

Figure 4. Electrophoretic mobility of 50 kDa protein in the presence and absence of reducing agent, 2-mercaptoethanol. Lane 1, broad M.W. standards; Lane 2, sample with treatment buffer containing 2-mercaptoethanol; Lane 3, sample without 2-mercaptoethanol.

IV. Discussion

Several Gram-negative bacteria are known to utilize hemin as a sole source of iron. In addition to their using this molecule as an apparent source of iron, hemin-binding proteins have been identified in several of these species, including *Shigella flexneri*[21], *Bacteroides fragilis*[18], *Neisseria gonorrheae*[15], *Hemophilus influenzae*[14,16,29], *Treponema denticola*[11], and *Porphyromonas gingivalis*[10,20,30]. However, only a few of these proteins have been purified and characterized.

Bacterial growth and membrane protein expression which are regulated by iron (hemin) have been reported in several microorganisms[11,18,31-34]. Many of these proteins have been implicated as functional components of iron (hemin) uptake systems in these species. Although the requirement for hemin

1				5				
N -	Ser -	Ile -	Ile -	Thr -	Asp -	Val -	Tyr -	Ala -
10				15				
Arg -	Glu -	Val -	Leu -	Asp -	Ser -	Arg -	Gly -	

Table 1. N'-terminal amino acid sequence of 50 kDa protein

has been known for many years, little is known about the mechanism(s) by which *P. intermedia* binds and uptakes hemin into the cell. There have been no reports of purification and characterization of the hemin-binding protein from *P. intermedia*.

The results presented in this report confirm that the availability of hemin influences the expression of a selected membrane protein as well as the growth rate of *P. intermedia* ATCC 25611. We consider 50 kDa cell envelope associated protein to be a putative hemin-binding protein from *P. intermedia*. Its expression appeared to be tightly regulated by the level of hemin in the growth medium.

In summary, this study has demonstrated the presence of a 50 kDa putative hemin-binding protein whose expression is hemin regulated in *P. intermedia* ATCC 25611. Disulfide bonds were not present in this protein, and N'-terminal amino acid sequence was identical with Enolase from *Streptococcus intermedius*. To our knowledge, the study described here is the first to identify, purify, and biochemically characterize a putative hemin-binding protein from *P. intermedia*. Work is in progress to further characterize the molecular structure of this protein.

V. Conclusion

In the study presented here, we carried out the identification, purification, and characterization of a putative hemin-binding protein in *Prevotella intermedia*. The results presented in this report confirm that the availability of hemin influences the expression of a selected membrane protein as well as the growth rate of *P. intermedia* ATCC 25611. We consider 50 kDa cell envelope associated protein, whose expression is hemin regulated, to be a putative hemin-binding protein from *P. intermedia*. Disulfide bonds were not present in this protein, and N'-terminal amino acid sequence was identical with Enolase from *Streptococcus intermedius*. To our knowledge, the study described here is the first to identify, purify, and biochemically characterize a putative hemin-binding protein from *P. intermedia*.

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-국문초록-

*Prevotella intermedia*에서의 hemin 결합 단백질의 순수분리 및 특성분석

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본 연구는 hemin이 치주질환 주요 병인균주 중의 하나인 *Prevotella intermedia*의 성장 및 세포막 단백질의 발현에 미치는 영향을 규명하고, hemin 결합에 관여하는 것으로 추정되는 단백질의 순수분리 및 특성 분석을 위해 수행되었다. 본 연구의 결과에 의하면, hemin은 *P. intermedia*의 성장 및 세포막 단백질의 발현에 영향을 미쳐, hemin이 고갈된 조건에서 균주의 성장이 현저히 억제되었으며, 약 50 kDa의 세포막 단백질이 현저히 강화되어 발현되었다. 본 연구에서는 hemin 결합에 관여하는 것으로 추정되는 이 50 kDa의 세포막 단백질을 순수분리하였으며, N'-terminal 아미노산 분석을 수행한 결과 이 단백질은 *Streptococcus intermedius*의 Enolase와 아미노산 서열 및 분자량이 일치하였다. 한편, 이 단백질에는 disulfide bond가 존재하지 않았다. 본 연구는 *P. intermedia*에서의 porphyrin 생리 및 hemin 획득 기전을 밝히는데 있어 중요한 의의가 있으리라 사료된다.

주요어 : *Prevotella intermedia*, hemin, 균주성장, 세포막 단백질, hemin 결합단백질