

# Isolation and characterization of the haemin-binding proteins from *Neisseria meningitidis*

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**The mechanism of haem-iron acquisition in *Neisseria meningitidis* is poorly understood. Using haemin-agarose in a batch affinity chromatography method, two haemin-binding proteins of 97 and 50 kDa were isolated from total membranes derived from *Neisseria meningitidis* B16B6 grown under iron-deficient but not under iron-replete conditions. No binding proteins were affinity-purified when total membranes underwent limited proteolysis with trypsin, suggesting a haem-protein interaction. When biotinylated human haemoglobin was used as the affinity ligand, proteins of identical molecular mass were isolated. Detection of haemin-binding proteins in a whole cell binding assay demonstrated a surface-exposed location. Competitive binding studies indicated that this haem-protein interaction was specific, because only haemin or human haemoglobin, but not cytochrome  $c_{111}$ , protoporphyrin IX, iron-loaded human lactoferrin, iron-loaded human transferrin or  $\text{Fe}(\text{NO}_3)_3$ , could abrogate binding. The presence of similar haemin-binding proteins in a limited survey of clinical meningococcal strains indicated that the expression of the haemin-binding proteins is not serogroup-specific.**

**Keywords:** *Neisseria meningitidis*, haemoglobin, haemin-binding proteins, meningococcal disease, iron

## INTRODUCTION

*Neisseria meningitidis*, a Gram-negative diplococcus, is an obligate human mucosal pathogen (DeVoe, 1982). Meningococcal disease, whose principal clinical expression is meningitis, remains a significant global public health problem (Peltola, 1983; Greenwood, 1984). Whereas most meningococcal cases cluster in small focal outbreaks in industrialized countries (Schlech *et al.*, 1985; Harrison & Broome, 1987), the epidemiology of meningococcal meningitis in sub-Saharan Africa is characterized by cyclical waves of epidemic disease that occur at 10–12 year intervals (Peltola, 1983; Greenwood, 1984).

Iron is a determinant of virulence in the meningococci. The addition of iron-containing compounds, such as iron dextran (Holbein, 1980), transferrin iron (Holbein, 1981), or haemoglobin (Brodeur *et al.*, 1985), enhances the lethality of meningococci in a murine model of infection. To acquire iron from the external environment, the meningococcus expresses surface-accessible iron-regulated proteins that demonstrate binding specificity to the host iron-containing compounds human lactoferrin

(Schryvers & Morris, 1988b) and human transferrin (Schryvers & Morris, 1988a). Receptors that exhibit such host specificity for these iron-sequestering glycoproteins have been identified in other pathogenic bacteria, such as *N. gonorrhoeae* (Lee & Schryvers, 1988; Lee & Bryan, 1989), *Haemophilus influenzae* type b (Schryvers, 1989), and *Actinobacillus pleuropneumoniae* (Gonzalez *et al.*, 1990). Cloning of the meningococcal (Irwin *et al.*, 1993; Legrain *et al.*, 1993) and gonococcal (Cornelissen *et al.*, 1992) transferrin receptor protein genes has permitted the construction of isogenic receptor mutants that are functionally incapable of binding and using human transferrin as an iron source. This genetic evidence supports the contention that in the pathogenic *Neisseriae*, specific interaction of transferrin with its cognate receptor is a necessary prelude for transferrin iron uptake.

In contrast, the mechanism of haem-iron acquisition in *N. meningitidis* is poorly understood. Using a solid phase dot enzyme assay, an outer membrane iron-regulated haemoglobin-binding protein has been described in the meningococcus (Lee & Hill, 1992). Competitive binding assays using haem and haem-containing compounds suggest that this binding protein recognizes the haem prosthetic group (Lee & Hill, 1992). This observation,

**Abbreviation:** HmBP, haemin-binding protein.

coupled with the recent demonstration of haemin-binding proteins (HmBPs) in *N. gonorrhoeae* (Lee, 1992a), suggests that haem-iron acquisition in the meningococcus may proceed via a siderophore-independent receptor-mediated pathway analogous to that of lactoferrin and transferrin iron, in which the initial event in haem-iron uptake involves the specific interaction with a cell surface HmBP(s). Therefore, the objective of this study was to isolate such surface accessible HmBPs in *N. meningitidis* and to determine the relationship of this component to the meningococcal haemoglobin-binding protein.

## METHODS

**Bacterial strains and growth conditions.** *N. meningitidis* B16B6, a standard serotyping strain, is a serogroup B, serotype 2A, strain kindly provided by A. Schryvers (University of Calgary, Canada). Clinical meningococcal isolates representing serogroups A, C, W-135 and X, were obtained from The Provincial Laboratory of Public Health for Southern Alberta, Calgary, Canada. Bacteria were maintained as stock cultures in skimmed milk at  $-70^{\circ}\text{C}$ , and, prior to use, organisms were grown on chocolate agar plates supplemented with 1% (v/v) CVA enrichment (Gibco) at  $35^{\circ}\text{C}$  in an atmosphere containing 5% (v/v)  $\text{CO}_2$ .

**Chemicals.** Human haemoglobin, equine cytochrome  $c_{111}$ , iron-loaded human lactoferrin, iron-loaded human transferrin, bovine haemin, protoporphyrin IX, ferric nitrate, EDDA (ethylenediamine di-*o*-hydroxyphenylacetic acid), guanidine HCl, and haemin-agarose were purchased from Sigma. All the iron- and haem-containing compounds were more than 95% pure according to the manufacturer. Streptavidin-horseradish peroxidase (SA-HRP) conjugate was purchased from BioCan Scientific. Desferrioxamine was obtained from Ciba-Geigy Canada. Trypsin was obtained from Sigma. With the exception of haemin, which was dissolved in a small volume of 10 mM NaOH, and protoporphyrin IX, which was solubilized in a small volume of 10 mM acetic acid, all the iron-containing compounds and haemoproteins were solubilized in deionized water immediately prior to use.

**Membrane preparation.** Crude total membranes were prepared as previously described (Lee & Hill, 1992) from cells grown in iron-replete [brain-heart infusion (BHI) broth (Difco)] and iron-limited (BHI broth rendered iron-deficient by the addition of an iron chelator, either EDDA or desferrioxamine, to a final concentration of 100  $\mu\text{M}$ ) conditions and from cells in which either bovine haemin (BHI broth supplemented with 12  $\mu\text{M}$  bovine haemin) or iron (BHI broth supplemented with 150  $\mu\text{M}$   $\text{Fe}(\text{NO}_3)_3$ ) served as the sole exogenous source of iron. The protein content was determined by the method of Lowry with bovine serum albumin as standard.

**Haemin affinity chromatography.** HmBPs were isolated by affinity-purification using haemin-agarose (Sigma) (Tsutsui & Mueller, 1982) as the affinity resin in a batch method as described previously (Lee, 1992a; Lee, 1992b). In brief, crude total membrane, adjusted to a final concentration of 1 mg  $\text{ml}^{-1}$  in 50 mM Tris/HCl and 1 M NaCl (pH 8.0), was incubated with 20  $\mu\text{mol}$  haemin-agarose at  $25^{\circ}\text{C}$  for 1 h. Binding was performed at high ionic conditions to prevent non-specific interaction between the proteins and the affinity gel. The membrane was solubilized with the addition of sodium EDTA (pH 8.0) and Sarkosyl NL30 to a final concentration of 10 mM and 0.75% (v/v), respectively, for 1 h at  $25^{\circ}\text{C}$ . The mixture was then centrifuged at 750  $g$  for 5 min, and the supernatant was

discarded. The pelleted ligand affinity resin was washed three times either with a high-salt wash, consisting of 50 mM Tris/HCl and 1 M NaCl (pH 8.0) or with a guanidine HCl wash, consisting of 500 mM, 1 M, or 2 M guanidine HCl in 50 mM Tris/HCl and 1 M NaCl (pH 8.0). Each of the above washes included sodium EDTA (5 mM final concentration, pH 8.0) and Sarkosyl NL30 (0.5% final concentration). After a final low-salt wash [50 mM Tris/HCl and 100 mM NaCl (pH 8.0)] without EDTA and detergent, bound proteins were eluted by the addition of 2% (w/v) SDS and 1% (v/v)  $\beta$ -mercaptoethanol in 500 mM Tris/HCl (pH 6.8) followed by boiling the affinity resin at  $100^{\circ}\text{C}$  for 5 min. The affinity gel was pelleted by centrifugation at 750  $g$  for 5 min. The supernatant was applied to a 12.5% polyacrylamide-SDS gel for analysis by SDS-PAGE.

**Haemoglobin affinity purification of haemoglobin-binding proteins.** Haemoglobin-binding proteins were isolated from iron-deficient total membranes by batch affinity purification analogous to the method used to isolate the meningococcal transferrin binding proteins (Schryvers & Morris, 1988a). Human haemoglobin was biotinylated with NHS-S-S-biotin as described previously (Lee & Hill, 1992). Retained proteins were eluted from the streptavidin-agarose affinity resin by the addition of 2% (w/v) SDS and 1% (v/v)  $\beta$ -mercaptoethanol in 500 mM Tris/HCl (pH 6.8) and were separated by SDS-PAGE.

**Whole-cell binding assay.** Organisms were grown on chocolate agar for 12–14 h at  $35^{\circ}\text{C}$  in the presence of 5% (v/v)  $\text{CO}_2$ . Cells were scraped from plates and suspended in BHI broth containing 100  $\mu\text{M}$  EDDA to an initial  $\text{OD}_{600}$  of 0.002, as measured using a Pye Unicam PU8800 spectrophotometer (Unicam Analytical). These cultures were shaken at  $35^{\circ}\text{C}$  in the presence of 5%  $\text{CO}_2$  or in an anaerobic chamber (model 800A, Anaerobe Systems, Santa Clara, CA, USA) rendered microaerophilic (5%  $\text{O}_2$ , 10%  $\text{CO}_2$ , 85%  $\text{N}_2$ ) until mid-exponential-phase growth was achieved ( $\text{OD}_{600}$  0.12–0.14). Samples were removed to inoculate fresh BHI broth with and without 100  $\mu\text{M}$  EDDA to a starting  $\text{OD}_{600}$  of 0.002. Following this second growth cycle to ensure depletion of internal iron stores, samples were again removed at mid-exponential phase and  $\text{OD}_{600}$  was adjusted to 0.25. After washing at  $4^{\circ}\text{C}$  in 50 mM Tris/HCl (pH 8.0), the cells were resuspended in 1 ml low-salt buffer and subjected to the affinity resin binding protocol as described above. Identical experiments were performed with 100  $\mu\text{M}$  desferrioxamine in lieu of EDDA as the iron chelator.

**Specificity of the HmBPs.** The binding specificity of the HmBPs was assessed by competition binding assays. Iron-deficient crude total membranes were preincubated for 1 h at  $25^{\circ}\text{C}$  with increasing concentrations of haemin, haem-containing and non-haem-containing compounds prior to haemin affinity chromatography.

**Proteolytic digestion of the *N. meningitidis* HmBPs.** Samples of crude total membranes prepared for the affinity-binding procedure were subjected to proteolytic digestion at  $37^{\circ}\text{C}$  with the addition of trypsin to a final concentration of either 25  $\mu\text{g ml}^{-1}$  or 50  $\mu\text{g ml}^{-1}$ . Proteolysis was stopped after 10 min with the addition of the serine protease inhibitor PMSF (BDH) to a final concentration of 200  $\mu\text{g ml}^{-1}$ . Affinity chromatography was performed on the resultant digests.

**Western blot analysis to detect haemoglobin-binding activity.** Haemin-affinity-purified proteins (25  $\mu\text{l}$ ) that were separated by SDS-PAGE were electroblotted at 10 V constant voltage for 12 h at  $4^{\circ}\text{C}$  onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P, 0.45  $\mu\text{m}$ , Millipore) by the method of Towbin *et al.* (1979) using a Bio-Rad MiniTransblot apparatus. The membrane was subsequently probed for the presence of haemoglobin-binding activity as previously de-

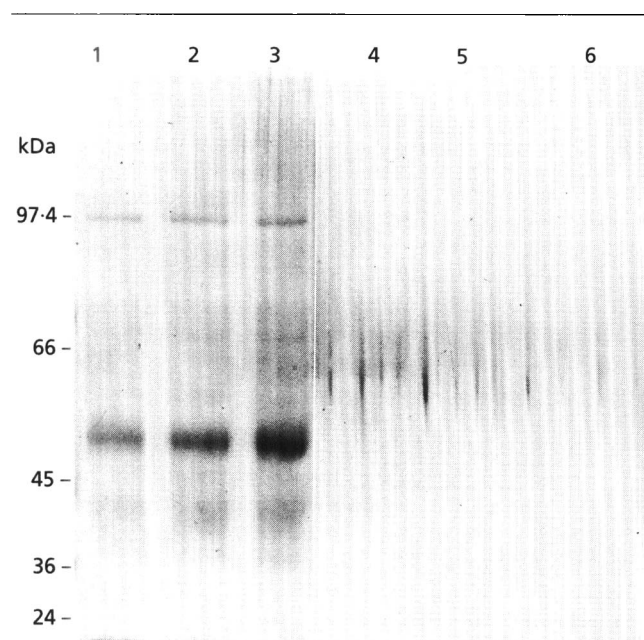
scribed (Lee & Hill, 1992). In brief, after blocking with 0.5% (w/v) skimmed milk in TBS (50 mM Tris/HCl, pH 7.5, 1 M NaCl), the membrane was incubated at 37 °C for 1 h with 50 nM biotinylated human haemoglobin followed by the addition of the SA-HRP conjugate at a concentration of 500 ng ml<sup>-1</sup>. The blot was developed with a chloronaphthol/hydrogen peroxide substrate mixture (HRP Reagent, Bio-Rad) for 10 min. The paper was washed with water to stop the reaction.

**Electrophoresis.** Proteins from affinity-purified total membranes or affinity-purified whole cells were analysed by SDS-PAGE with the discontinuous buffer system of Laemmli (1970). The resolving gel was 12.5% acrylamide and the stacking gel was 5% acrylamide with 0.8% bisacrylamide. Gels were electrophoresed at 150 V constant voltage for 1 h on a Bio-Rad minigel apparatus. Gels were stained with silver (Oakley *et al.*, 1980). Molecular masses were determined by using known proteins as standards.

## RESULTS

### Purification of HmBPs from *N. meningitidis* B16B6

Two HmBPs of molecular mass 97 and 50 kDa were isolated by haemin affinity chromatography (Fig. 1, lane 1). The 97 kDa protein on occasion migrated as an apparent doublet under reducing SDS-PAGE. However,



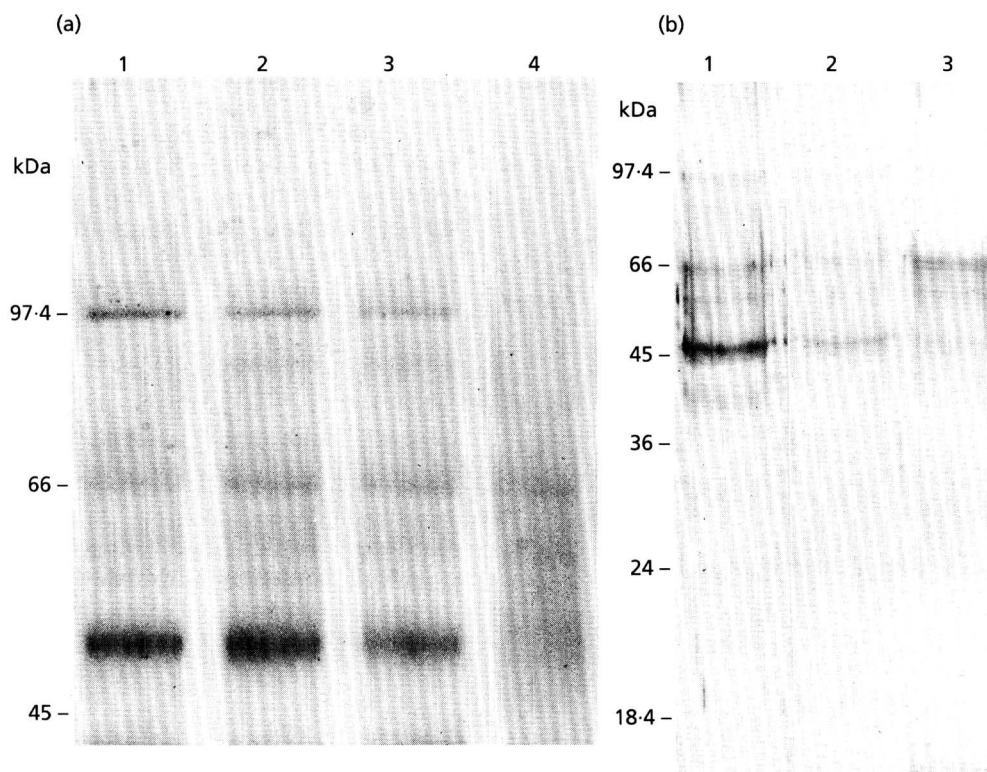
**Fig. 1.** Batch affinity isolation of HmBPs and regulation of expression of the HmBPs from *N. meningitidis* B16B6. The HmBPs were affinity-purified as described in Methods from total membranes derived from bacteria grown under iron-limiting conditions (lanes 1 and 2) or from iron-deficient whole cells (lane 3). Iron-restriction was imposed by the addition of either 100  $\mu$ M desferrioxamine (lane 1) or 100  $\mu$ M EDDA (lanes 2 and 3) to the bacterial cultures. HmBPs were affinity-isolated from membranes prepared from cells grown under iron-sufficient conditions (lane 4) or from cells grown under iron-restricted conditions in medium supplemented with either 150  $\mu$ M Fe(NO<sub>3</sub>)<sub>3</sub> (lane 5) or 12  $\mu$ M bovine haemin (lane 6). The eluted proteins were separated by SDS-PAGE and the gel was stained with silver. Sizes of protein standards are shown on the left.

this protein resolved to a single band when the amount of affinity-purified total membranes loaded onto the polyacrylamide gel was progressively reduced (data not shown). Two other observations also suggested that the appearance of the doublet could be attributed to sample overload and not due to a second affinity-purified protein co-migrating with the 97 kDa HmBP. Firstly, neither the addition of fresh  $\beta$ -mercaptoethanol to the samples nor the substitution of dithiothreitol as the reducing agent altered the migration characteristics of the 97 kDa doublet (data not shown). Secondly, analysis of the affinity-purified proteins using a 4–20% polyacrylamide gradient in the separating gel did not result in a further separation of the apparent doublet (data not shown). An alternative explanation for the anomalous migratory and tinctorial characteristics of the 97 kDa protein may be the presence of an attached carbohydrate and/or phospholipid moiety. Another meningococcal outer membrane iron-binding receptor, *tbp2*, has been shown to be a lipoprotein (A. B. Schryvers, unpublished study). Additionally, partial proteolytic degradation of the 97 kDa HmBP could account for the doublet appearance.

The HmBPs were only detected under conditions of iron limitation (Fig. 1, lane 2). Their expression was independent of the iron chelator used to impose iron depletion (Fig. 1, lane 1). In contrast, no HmBPs were isolated from total membranes grown under iron-rich conditions (Fig. 1, lane 4). Similarly, when iron-restriction was reversed with the addition of 150  $\mu$ M Fe(NO<sub>3</sub>)<sub>3</sub> (Fig. 1, lane 5), or when 12  $\mu$ M bovine haemin was provided as the sole exogenous iron source (Fig. 1, lane 6), no HmBPs were affinity-purified from these membranes. These results indicated that the expression of the HmBPs is iron-regulated. The inability to isolate HmBPs from total membranes grown in the presence of bovine haemin as the sole iron source suggests that haemin may also regulate the expression of the HmBPs. Alternatively, the presence of haemin in the total membrane mixture may have competitively inhibited binding of the HmBPs to the affinity gel, and precluded their subsequent isolation.

No HmBPs could be affinity-isolated from iron-sufficient cultures grown either under microaerophilic conditions or at 25 °C (data not shown). These results suggested that the expression of the HmBPs is neither governed by oxygen deprivation nor by slow growth rate.

The HmBPs were absent when the affinity resin alone, without applied membranes, was subjected to the affinity protocol, indicating that the HmBPs are neither degradation products nor derivatives of the agarose matrix (data not shown). The binding of the HmBPs to their ligand is a conformation-dependent process, since detergent solubilization of the membranes with the ionic detergent SDS prior to binding to the matrix-bound haemin also prevented isolation of the HmBPs (data not shown). Two explanations may account for the protein bands of 60–70 kDa seen in the gels. Firstly, they may represent contaminating proteins present in the  $\beta$ -mercaptoethanol used in the elution buffer, as these bands were only present when this reducing agent was included



**Fig. 2.** (a) Haemin competition binding of the HmBPs. Bovine haemin at 0  $\mu$ M (lane 1), 1  $\mu$ M (lane 2), 10  $\mu$ M (lane 3) and 1 mM (lane 4) was incubated for 1 h at 25  $^{\circ}$ C with 1 mg total membranes prepared from iron-limited cultures of *N. meningitidis* B16B6. The HmBPs from these preparations were affinity-purified and separated by SDS-PAGE. The gel was stained with silver. Sizes of protein standards are shown on the left. (b) Haemoglobin competition binding of the HmBPs. Human haemoglobin at 0  $\mu$ M (lane 1), 1  $\mu$ M (lane 2) and 10  $\mu$ M (lane 3) was incubated with a constant amount (1 mg) of iron-limited total membranes of *N. meningitidis* B16B6 for 1 h at 25  $^{\circ}$ C. The HmBPs were affinity-isolated and separated by SDS-PAGE. The resultant gel was stained with silver. Sizes of protein standards are shown on the left.

in the elution buffer (data not shown) and were absent when it was omitted (data not shown) (Tasheva & Dessev, 1983). Secondly, because such bands were occasionally seen in samples electrophoresed using other reducing agents, such as dithiothreitol (data not shown), they may represent artifactual keratin protein contaminants (Ochs, 1983; Merrill, 1990).

To determine which component of the HmBPs bound directly to haemin, the haemin affinity resin containing the bound proteins was subjected to washes of increasing stringency with guanidine HCl. No quantitative or qualitative differences were seen in the HmBPs that were eluted after washing with 500 mM, 1 M or 2 M guanidine HCl (data not shown), indicating that there was no preferential retention of either HmBP on the affinity matrix.

### Cellular location of the HmBPs

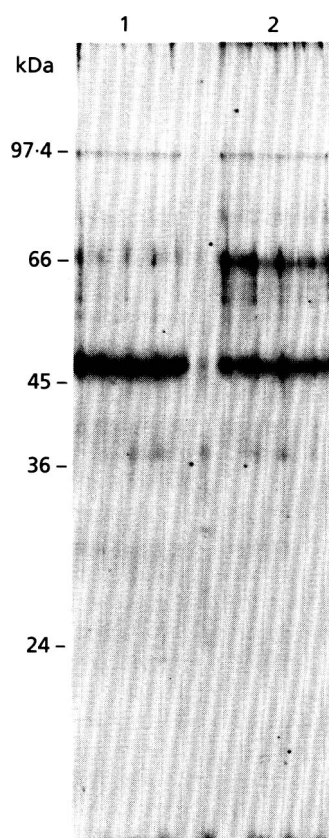
Isolation of HmBPs from whole-cells grown under iron-limited conditions (Fig. 1, lane 3) but not from whole-cells grown under iron-rich conditions (data not shown) indicated that the HmBPs were both surface-exposed and iron-regulated. The conditions used in the whole-cell assay have previously been shown to maintain whole-cell integrity (Lee & Hill, 1992). The minimal cell lysis present excluded significant contamination of the whole-

cell samples with intracellular sequestered haem-containing proteins, such as cytochrome proteins.

### Specificity of the meningococcal HmBPs

The specificity of the HmBPs for haemin was addressed in a series of competition binding experiments. The binding of the HmBPs to the immobilized haemin was specifically blocked by unlabelled haemin in a concentration-dependent manner (Fig. 2a, lanes 1–3). Haemin concentrations in excess of 10  $\mu$ M markedly diminished binding of the HmBPs to the haemin-agarose. This binding specificity extended to human haemoglobin. The presence of this haemoprotein at concentrations above 1  $\mu$ M also reduced adsorption of the HmBPs to the affinity gel (Fig. 2b, lanes 1–3). The aggregation of tetrapyrroles in aqueous solutions at physiological pH (Brown *et al.*, 1976; Muller-Eberhard & Nikkila, 1989) may account for the diminished ability of haemin relative to haemoglobin to inhibit binding competitively. This property could result in the formation of stacked haemin aggregates that would reduce the effective concentration of binding.

In contrast, no binding inhibition was observed when iron-restricted total membranes prepared from *N. meningitidis* B16B6, or from a serogroup C meningococcal isolate, were preincubated with another haem-containing protein,



**Fig. 3.** Haemoglobin-binding proteins affinity-purified from *N. meningitidis* B16B6. Haemoglobin-binding proteins were isolated from iron-limited crude total membranes by batch haemoglobin-affinity chromatography as described in Methods (lane 1). The same membranes were subjected to haemin-affinity purification (lane 2). Retained proteins were eluted and separated by SDS-PAGE. The gel was stained with silver. Sizes of protein standards are shown on the left.

equine cytochrome  $c_{111}$  (data not shown). The functional correlates of this binding specificity demonstrate this correspondence and have been shown in a previous study (Lee & Hill, 1992). At concentrations of 10  $\mu$ M, protoporphyrin IX, the immediate precursor of haem in the haem biosynthetic pathway, was incapable of blocking binding (data not shown). Two non-haem iron-containing glycoproteins, iron-loaded human lactoferrin and iron-loaded human transferrin, and  $\text{Fe}(\text{NO}_3)_3$  at concentrations of 10  $\mu$ M also did not competitively inhibit binding of the HmBPs to the haemin-bound polysaccharide matrix (data not shown).

#### Proteolytic assays

No HmBPs were purified when total membranes were incubated with either 25 or 50  $\mu\text{g ml}^{-1}$  of trypsin for 30 min at 37  $^\circ\text{C}$  prior to affinity isolation (data not shown). This observation indicates that either a protein determinant(s) is necessary for the interaction of the HmBPs with their ligand or that preservation of the surrounding protein architecture is necessary for binding.

#### Isolation of HmBPs from other meningococcal strains

HmBPs with molecular masses similar to those found in *N. meningitidis* B16B6 were affinity-purified from iron-limited total membranes prepared from representative strains belonging to serogroups A, C, X and W-135 (data not shown). This result suggests that these polypeptides are structurally and functionally conserved among clinically significant meningococcal serogroups.

#### Isolation of haemoglobin-binding protein(s) from *N. meningitidis* B16B6

Two haemoglobin-binding proteins (Fig. 3, lane 1) corresponding to the molecular masses of the HmBPs (Fig. 3, lane 2) were isolated from iron-limited total membranes by haemoglobin-affinity chromatography. This result suggests that a structural homology exists between the haemoglobin- and haemin-binding proteins. In an attempt to demonstrate a functional identity between the two binding proteins, PVDF membranes containing electroblotted SDS-PAGE separated HmBPs, and nitrocellulose/cellulose acetate paper (Immobilon-NC, 0.45  $\mu\text{m}$  HAHY paper, Millipore) containing 1  $\mu\text{l}$  samples of immobilized HmBPs, were probed with biotinylated human haemoglobin. No haemoglobin-binding activity was seen in either blot (data not shown). The apparent conformation-dependent interaction between the HmBPs and their ligand (see above) provides an explanation for the lack of functional haemoglobin-binding activity in either denatured preparation.

#### DISCUSSION

Haemin and haem-containing compounds can promote the *in vitro* growth of many human bacterial pathogens (Perry & Brubaker, 1979; Helms *et al.*, 1984; Stull, 1987; Pidcock *et al.*, 1988; Stoebner & Payne, 1988; Daskaleros *et al.*, 1991; Lee, 1991). For *Porphyromonas gingivalis* (Bramanti & Holt, 1992a, b), *N. gonorrhoeae* (Lee, 1992a), and *H. influenzae* type b (Lee, 1992b), this ability to use haem and haemoproteins as the sole exogenous iron source has been attributed to the presence of a surface-exposed outer membrane haemin-binding protein(s) whose expression is governed by iron. In part because of the biochemical constraints imposed by free haem (Muller-Eberhard & Nikkila, 1989; Vincent, 1989), it has been proposed that the specific binding of haem to these outer membrane constituents is a necessary prelude for the uptake of haem-iron (Bramanti & Holt, 1992a; Lee, 1992a, b). The identification of two meningococcal surface-exposed iron-regulated haemin-binding proteins in this study is consistent with the supposition that haem-iron uptake may proceed via such a receptor-mediated process in *N. meningitidis*. The recent genetic demonstrations that haemin uptake in *Yersinia enterocolitica* (Stojiljkovic & Hantke, 1992) and in *Vibrio cholerae* (Henderson & Payne, 1993) requires a haemin-specific iron-regulated outer membrane receptor support this contention.

Using a solid-phase dot enzyme binding assay, in which biotinylated human haemoglobin was used to probe iron-

limited meningococcal whole-cells and total membranes for haemoglobin-binding activity, a previous investigation reported the presence of an outer membrane haemoglobin-binding protein(s) in *N. meningitidis* (Lee & Hill, 1992). Several lines of evidence suggest that the HmBPs isolated in this study represent these putative haemoglobin-binding proteins. Firstly, a functional identity exists between the two binding proteins, since irrespective of whether haemin or haemoglobin is used as the affinity ligand, proteins of the same size are affinity-purified from iron-deficient total membranes (Fig. 3, lanes 1 and 2). Secondly, despite quantitative differences attributable to methodological considerations, the binding specificities of the two binding proteins display a reciprocal relationship. Human haemoglobin is capable of inhibiting binding of the HmBPs to haemin-agarose (Fig. 2b, lanes 1–3), whereas haemin competitively inhibits haemoglobin-binding activity in a concentration-dependent manner (Lee & Hill, 1992). As previously proposed (Lee & Hill, 1992), this latter property suggests that the haem prosthetic group in haemoglobin is the ligand recognized by the haemoglobin-binding protein(s). Thirdly, the haemin- and haemoglobin-binding proteins share qualitatively similar inhibition profiles. Cytochrome  $c_{111}$ , protoporphyrin IX, iron-loaded human lactoferrin, iron-loaded human transferrin, and iron cannot competitively block binding (data not shown) (Lee & Hill, 1992). Lastly, although it is unknown whether both polypeptides interact directly with haemin, the presence of two meningococcal haemoglobin-binding proteins is compatible with the two component model of binding (Smith & Ledford, 1988) as predicted by Scatchard plot analysis (Scatchard, 1946) of the haemoglobin-binding proteins (Lee & Hill, 1992).

All these observations suggest that the existence of discrete haemoglobin-binding proteins that are distinct from the HmBPs identified in this study may not be warranted. This evidence also implies that haemoglobin-binding activity in the meningococcus functions as a surrogate marker for the HmBPs. The suggestion that haem- and haemoglobin-iron uptake may be mediated by binding to a common receptor is reinforced by the observation in *V. cholerae*, in which iron utilization from haem and haemoglobin appears to require the synthesis of at least two proteins, one of which is an iron-regulated outer membrane protein proposed to act as a haem receptor (Henderson & Payne, 1993).

The HmBPs in the pathogenic *Neisseria* species share several common properties, leading to the premise that the meningococcal polypeptides may represent the structural and functional homologues of the gonococcal HmBPs. The size of the meningococcal HmBPs closely resembles their gonococcal counterparts, in which the corresponding HmBPs are 97 kDa and 44 kDa (Lee, 1992a). Both meningococcal and gonococcal HmBPs possess quantitatively similar competitive inhibition profiles for haemin, haemoglobin, and for the non-haem-containing glycoproteins hLf and hTf (Lee, 1992a). The discrepancy in the ability of cytochrome  $c_{111}$  to inhibit binding competitively is unexplained but may reside in

strain differences as only one gonococcal isolate was examined (Lee, 1992a). These analogies in the HmBPs are reminiscent of comparisons between another iron-repressible neisserial outer membrane protein receptor, the meningococcal and gonococcal transferrin (Tf) receptor, in which similarities are present not only in the structure and functional specificity of the meningococcal and gonococcal Tf receptors (Cornelissen *et al.*, 1992), but also in the genomic organization and nucleotide sequences of their respective Tf receptor genes (Legrain *et al.*, 1993).

The mechanisms responsible for the apparent similarities in structure and function of the HmBPs, and of the Tf receptor, in the pathogenic *Neisseria* are unknown. The observations that *Neisseria* species are naturally competent for transformation and that the selectivity for DNA uptake is restricted at a genus, but not at a species level (Dougherty *et al.*, 1979), lead to the intriguing speculation that interspecies transformation-mediated localized re-combinational events may account for such similarities. The gonococcus and meningococcus occupy the same ecological niches (Faur *et al.*, 1975), providing the opportunities for such a horizontal exchange of genetic material. This molecular mechanism underlies the mosaic structure in the penicillin-binding protein 2 genes (*penA*) from *N. gonorrhoeae* and *N. meningitidis* (Spratt, 1988; Spratt *et al.*, 1989) whereby the *penA* genes in penicillin-resistant organisms contain blocks of DNA recruited from either *N. flavescens* or *N. cinerea* (Spratt *et al.*, 1992). However, there is currently no genetic evidence to attribute this mechanism as being responsible for the apparent structural and functional homology of the HmBPs in the gonococcus and meningococcus.

It is becoming more apparent that the meningococcus and gonococcus express a diverse array of iron-regulated surface-exposed binding proteins that interact specifically with host iron-containing compounds (Lee & Schryvers, 1988; Schryvers & Morris, 1988a, b; Lee & Bryan, 1989; Lee, 1992a). The iron-regulated expression of surface-accessible haemin-specific binding proteins in the meningococcus described in this study supports this supposition. Although the contribution from exogenous siderophore sources is unknown (West & Sparling, 1987), these pathogenic bacteria may satisfy their iron requirements by acquiring this critical element exclusively through iron uptake systems mediated by the specific binding of host iron-containing proteins to these receptors. However, the role of these receptors, and of the HmBPs in particular, in the pathogenesis of meningococcal and gonococcal disease will await further rigorous genetic analysis.

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