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Affinity, Conservation, and Surface Exposure of Hemopexin-Binding Proteins in *Haemophilus influenzae*

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Haemophilus influenzae **can acquire heme from hemopexin for use as a source of both essential porphyrin and iron. In classical ligand-binding studies, we observed time-dependent, saturable, and displaceable binding of human ^{125}I-labelled hemopexin to intact cells of *H. influenzae* type b (Hib) strain 760705 grown in an iron-restricted medium. From these experiments, which demonstrate that hemopexin associates with a single class of binding site, the affinities (K_ds) and receptor numbers were calculated for heme-hemopexin (K_d, 205 nM; 3,200 receptors per cell) and apohemopexin (K_d, 392 nM; 4,400 receptors per cell). Thus, Hib expresses a specific hemopexin receptor which shows some preference for the heme-protein complex. Affinity chromatography on hemopexin-Sepharose 4B of detergent-solubilized membranes from Hib strain 760705 results in the copurification of three proteins with molecular masses of 57, 38, and 29 kDa. Trypsinization of whole cells of Hib 760705 abolishes hemopexin binding and correlates with the disappearance of the 57-kDa hemopexin-binding protein and appearance of a 52-kDa species which does not bind either hemopexin in ligand blot assays or a monoclonal antibody (MAbT11-30) raised against the 57-kDa protein. From immunoblotting assays and NH_2-terminal amino acid sequence analysis, the 38-kDa protein isolated following hemopexin affinity chromatography was identified as the porin protein P2. These data, taken together with the receptor-binding studies which support a single class of hemopexin-binding site, suggest that P2 and the 29-kDa protein function as accessory proteins to the 57-kDa hemopexin-binding protein to facilitate the uptake of heme from receptor-bound hemopexin. To determine whether hemopexin binding and the 57-kDa protein are conserved in *Haemophilus* strains, whole-cell dot blots and immunoblots of the outer membrane proteins prepared from strains belonging to each of 21 different Hib outer membrane protein subtypes, six nontypeable strains, and five *Haemophilus parainfluenzae* strains were probed with either hemopexin or MAbT11-30. Only the *H. parainfluenzae* strains which lack the 57-kDa protein do not bind hemopexin. Since *H. influenzae* has also been shown to produce a soluble 100-kDa hemopexin-binding protein, cell-free culture supernatants were also examined for the presence of this protein. Apart from Hib 760705 and *H. parainfluenzae*, the 100-kDa hemopexin-binding protein was detected in all the other *Haemophilus* strains. The abilities of Hib 760705 to both bind and acquire heme from hemopexin without expressing a 100-kDa soluble hemopexin-binding protein show that in strain 760705, this 100-kDa protein is not essential for the utilization of heme from hemopexin.**

Haemophilus species, including *H. influenzae* and *H. parainfluenzae*, are common commensals of the human upper respiratory tract. Although *H. influenzae* constitutes only a relatively small proportion of the commensal flora, it is much more frequently responsible for clinically significant infections than any other *Haemophilus* species (1, 34). *H. influenzae* can be readily distinguished from *H. parainfluenzae* by an absolute requirement for porphyrin, since it lacks the enzymes necessary to convert δ-aminolevulinate to protoporphyrin IX (PPIX), the intermediate biosynthetic precursor of heme. Most *H. influenzae* strains can synthesize heme from PPIX, since they express a ferrochelatase which catalyzes the insertion of a single iron atom into the PPIX macrocycle (39). Thus, in conventional iron-plentiful laboratory media, PPIX can substitute for heme (40) and promote the growth of *H. influenzae*.

In mammalian extracellular body fluids, the plasma proteins transferrin and hemopexin ensure that there is virtually no free iron or heme, thus providing an environment unsuitable for microbial growth (6, 23, 41, 44). However, *H. influenzae* has evolved mechanisms to overcome this nutritional restriction and can sequester protein-bound iron and heme from iron-transferrin, hemoglobin, and even haptoglobin-hemoglobin, heme-hemopexin, and heme-albumin complexes (7, 16, 17, 20, 29, 42, 43). Since commensal haemophili, including *H. parainfluenzae* and *H. paraphrophilus*, are unable to bind or acquire iron from transferrin or heme from hemoglobin-haptoglobin or heme-hemopexin (7, 16, 17, 42), these heme- and iron-scavenging mechanisms are considered to contribute to the virulence of *H. influenzae*.

For *H. influenzae*, both type b (Hib) and nontypeable strains acquire iron from transferrin via a mechanism which depends on the direct interaction between a cell surface receptor consisting of two iron-regulated transferrin-binding proteins (transferrin-binding proteins 1 and 2 [Tbp1 and Tbp2]) and the iron-binding glycoprotein (8, 17, 28, 41). Iron availability has also been shown to influence the binding of hemopexin to Hib, since binding was apparent only when the bacteria were cultured in a medium supplemented with PPIX and the iron chelator ethylenediamine-di-*o*-hydroxyphenyl acetic acid (EDDA) (43). Hib grown in iron-replete media containing

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either PPIX or heme did not bind hemopexin (43). Using hemopexin affinity chromatography of detergent-solubilized membranes, Wong et al. (43) isolated three proteins of 57, 38, and 29 kDa from three different Hib strains (760705, DL42, and Eagan); the 57-kDa protein could be renatured to bind hemopexin after Western blotting (immunoblotting). Attempts to elute selectively any of the three proteins from the hemopexin affinity resin with denaturants were not successful, suggesting that they may form part of a hemopexin receptor-accessory protein complex (43).

Using a colony blot assay, Hanson et al. (7) also demonstrated that *H. influenzae* could bind hemopexin. This hemopexin binding was associated with a 100-kDa protein, the loss of which by mutation correlated with an inability to bind or utilize heme-hemopexin when the mutant was grown in an iron-replete, heme-deficient medium (7). Furthermore, expression of this 100-kDa protein now termed HxuA (3) was enhanced after anaerobic growth of *H. influenzae* in the absence of heme compared with aerobic growth in the presence of heme (7, 43). Interestingly, Hib strain 760705 does not express a 100-kDa protein (7, 43) but binds hemopexin after aerobic growth in an iron-restricted medium (43). HxuA has recently been shown to be a secreted protein released into the culture supernatant (3). Thus, the relative contributions of these two apparently distinct mechanisms to the binding of hemopexin and uptake of protein-bound heme are not yet evident. Since Hib strain 760705 lacks the 100-kDa protein, in this study we sought to (i) confirm the existence of a specific saturable surface receptor for hemopexin in this strain; (ii) assess the relative contributions of the 57-, 38-, and 29-kDa proteins isolated by affinity chromatography to hemopexin binding; and (iii) establish whether both the 57- and 100-kDa hemopexin-binding proteins are conserved in Hib strains belonging to each of the recognized outer membrane subtypes, in nontypeable strains, and in *H. parainfluenzae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Hib strains representing each of the 21 recognized outer membrane subtypes (1L, 1H, 2L, 2H, 3L, 4H, 5L, 6U, 7H, 8H, 9L, 10H, 11L, 12U, 13L, 14L, 15L, 16L, 17H, 18L, and 19H) (2, 36) were kindly provided by Loek van Alphen (Department of Microbiology, University of Amsterdam, Amsterdam, The Netherlands). Hib strain 760705 (outer membrane subtype 1L), which is representative of the majority of invasive disease isolates in Europe, was isolated from the cerebrospinal fluid of a patient with meningitis (36, 37). Six nontypeable *H. influenzae* strains (2444, 2580, 2584, 2612, 2753, and 2844) were obtained from R. G. Finch (Department of Microbial Diseases, City Hospital, Nottingham, United Kingdom); *H. parainfluenzae* P205 has been described previously (40), and *H. parainfluenzae* 8PS, 16U, 21E, and DTL1 were kindly provided by R. Quentin (Laboratoire de Bactériologie, Centre Hospitalier Régional and Universitaire de Tours, Hôpital Bretonneau, Tours, France). Bacteria were grown routinely on chocolate blood agar plates at 37°C. Strains were stored at −70°C in brain heart infusion broth containing 20% (vol/vol) glycerol. For experiments in iron-replete liquid medium, bacteria were grown with aeration at 37°C in brain heart infusion (BHI) broth supplemented with 2 μg of NAD per ml and 0.5 μg of PPIX per ml (sBHI). Iron restriction was achieved by the addition of EDDA to sBHI to a final concentration of 100 μM (16).

Isolation and characterization of hemopexin. Human hemopexin was purified from fresh human serum by the method of Vretblad and Hjorth (38) as described previously (43). The purity of the isolated protein was greater than 97%, as determined by overloading samples and analyzing by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The heme-hemopexin complex was made by the addition of 1:1.1 molar equivalents of heme to ice-cold hemopexin solution. After 20 min of incubation, the extent of complex formation was assessed from the absorbance spectrum (250 to 450 nm) (15). The complexes were dialyzed at 4°C overnight against an appropriate buffer or water prior to lyophilization and storage at 4°C.

Preparation of labelled human hemopexin. Apo- and heme-hemopexin were labelled with either biotin or ^{125}I. Biotinylated hemopexin was prepared with *N*-hydroxysuccinimido-biotin (Pierce, Rockford, Ill.) as described by Wong et al. (43) and after conjugation stored at 4°C. Hemopexin was iodinated as described by Modun et al. (13) for labelling transferrin. Briefly, 200 μl of a 100-μg/ml solution of iodogen (Pierce, Rockford, Ill.) in dichloromethane was added to a 4-ml test tube. The dichloromethane was allowed to evaporate by rotating the test tube in a water bath at 37°C. Either apohemopexin or heme-hemopexin (300 μl of a 1-μg/ml solution in phosphate-buffered saline [PBS]) and approximately 6 MBq of carrier-free sodium ^{125}I (Amersham International plc) were added to each iodogen-coated tube. The mixture was incubated with agitation at room temperature for 15 min, and the unincorporated ^{125}I was removed by gel permeation chromatography with Sephadex G-25 (Pharmacia) preequilibrated with PBS containing 0.25% (wt/vol) hemopexin.

The hemopexin affinity resin was prepared as previously described by coupling either apohemopexin or heme-hemopexin to CNBr-activated Sepharose 4B (43).

Whole-cell hemopexin-binding assays. The ability of whole bacterial cells to bind hemopexin was evaluated by a dot enzyme assay as described previously (43). Briefly, bacteria were resuspended in 50 mM Tris-HCl (pH 7.4) to give a suspension with an optical density at 600 nm of 1.0 of which 5 μl was spotted onto a nitrocellulose membrane (pore size, 0.45 μm). After drying at room temperature for 10 min, dot blots were blocked with 1% skim milk in 50 mM Tris-HCl (pH 7.4), and then incubated with biotinylated heme-hemopexin (1 μg/ml). For some experiments, whole bacterial cells in suspension were treated with the proteolytic enzyme trypsin (0 to 25 μg/ml; Sigma bovine type VIII) for 45 min at 37°C. Proteolysis was stopped by the addition of soybean trypsin inhibitor (15 μg/ml; Sigma), and the cells were then washed and either dot blotted or used to prepare membranes as described below. Cell-bound biotinylated hemopexin was detected with a streptavidin-horseradish peroxidase conjugate (2 μg/ml) and then developed with 25 μg of 4-chloro-1-naphthol per ml and 0.01% (vol/vol) H_2O_2 in 50 mM Tris-HCl (pH 7.4).

For determination of the kinetics of ^{125}I-hemopexin binding, *H. influenzae* 760705 (10^8 CFU), in a final assay volume of 1 ml, grown in sBHI with EDDA, was incubated with either apo– or heme–^{125}I-hemopexin (5.1 nM in PBS) for intervals of 5, 10, 20, 30, 60, and 90 min at 37°C in a water bath. Bacteria were then pelleted, washed five times by resuspension in PBS, and transferred to a fresh microcentrifuge tube, and the amount of cell-associated ^{125}I-hemopexin was determined with an LKB 1282 Compugamma counter (Pharmacia LKB, Uppsala, Sweden). Specific binding was defined as the difference between the amount of ^{125}I-hemopexin bound in the absence and presence of a 100-fold excess of the unlabelled ligand. Receptor number and affinity (K_d) were calculated from competitive binding experiments in which the binding of a single concentration of radioligand was inhibited by increasing concentrations of the same unlabelled ligand by using hemopexin as the radioligand and competitor as described by De Blasi et al. (5). By using the curve-fitting program Graph Pad Inplot (ISI Inc.), the curves obtained were fitted to the four-parameter logistic equation $y = A + \{(B - A)/[1 + (10^C/10^x)^D]\}$, where A is the bottom plateau of the curve, B is the top plateau, x is the value in the middle of the curve, and D is the Hill coefficient. For these experiments, Hib strain 760705 was incubated for 30 min at 37°C with either 8.5 nM apo–^{125}I-hemopexin or heme–^{125}I-hemopexin in the presence of a range of concentrations of unlabelled human hemopexin (0 to 3 μM).

Preparation of membranes and hemopexin-binding proteins. After the bacterial cells were harvested, they were resuspended in 50 mM Tris-HCl buffer (pH 7.4) containing 50 μg of phenylmethylsulfonyl fluoride per ml and 50 μg of benzamidine per ml and lysed by sonication. Unbroken cells were removed by centrifugation at 5,000 × *g* for 5 min, and whole-cell envelopes were collected by centrifugation (40,000 × *g* for 60 min at 4°C). *H. influenzae* outer membranes were prepared by treating cell envelopes with 2% (vol/vol) Triton X-100 as described by van Alphen et al. (36, 37). For Hib strain 760705, membrane-associated hemopexin-binding proteins were isolated by batch adsorption with hemopexin-Sepharose 4B affinity resin as described previously (43). Briefly, cell envelopes were resuspended to a protein concentration of 5 μg/ml in 50 mM Tris-HCl (pH 8.0) containing 100 mM NaCl and 5 mM CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate} (Novobiochem). After incubation for 60 min on a rotary shaker at room temperature, insoluble material was removed by centrifugation and the CHAPS-soluble fraction was used either directly for SDS-PAGE and immunoblotting or for affinity purification on hemopexin-Sepharose 4B. For some experiments, whole Hib cells were incubated with trypsin as described prior to sonication and solubilization with CHAPS. The 100-kDa soluble hemopexin-binding protein was prepared from cell-free supernatants of *H. influenzae* strains essentially by the method of Cope et al. (3).

SDS-PAGE and immunoblotting. Proteins were analyzed by SDS-PAGE on 10% polyacrylamide separating gels (approximately 2 μg of protein was loaded per lane) and immunoblotting as described previously (43). Blots were probed either with MAbT11-30, which recognizes the 57-kDa hemopexin-binding protein as well as a 30-kDa protein (43), or with a monospecific polyclonal antiserum to the *H. influenzae* porin protein P2 (kindly provided by T. F. Murphy, Division of Infectious Diseases, Department of Medicine, State University of New York at Buffalo). Bound antibodies were detected with a protein A-horseradish peroxidase conjugate (Sigma) and developed with 4-chloro-1-naphthol as described above. SDS-polyacrylamide gels were stained with Coomassie brilliant blue.

Protein sequence analysis. Affinity-isolated hemopexin-binding proteins were resolved by SDS-PAGE with 10% acrylamide slab gels. Proteins were then transferred electrophoretically to polyvinylidene difluoride membranes (Problot; Applied Biosystems, Warrington, United Kingdom) for determination of their NH_2-terminal amino acid sequences by solid-phase direct protein sequencing (Applied Biosystems Protein Sequencer model 473A). Amino acid sequences

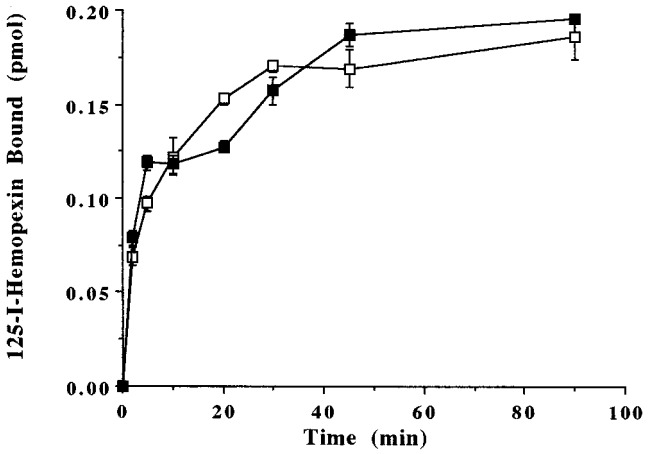

FIG. 1. Time course of human heme–^{125}I-hemopexin (■) and apo–^{125}I-hemopexin (□) binding to Hib strain 760705. After overnight growth in sBHI containing 100 μM EDDA, bacteria were harvested, washed, resuspended in PBS (10^8 cells per ml) containing 5.1 nM ^{125}I-labelled hemopexin, and incubated at 37°C. At intervals of 2, 5, 10, 20, 30, 45, and 90 min, bacteria were pelleted and the amount of cell-associated ^{125}I-hemopexin was determined. Data presented are the means of three independent experiments ± standard deviations.

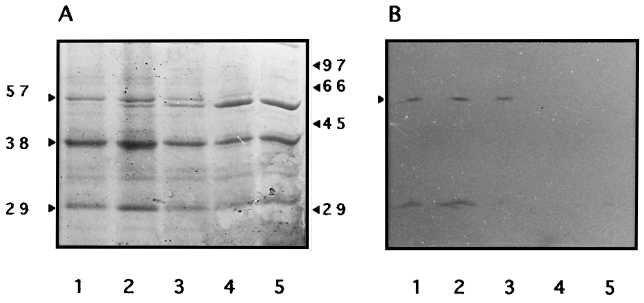

FIG. 3. SDS-polyacrylamide gel (A) and immunoblot (B) showing the influence of trypsin on the hemopexin receptor protein complex of Hib strain 760705. Whole bacterial cells were incubated without (lane 1) or with trypsin (at 0.1 [lane 2], 1.0 [lane 3], 2.5 [lane 4], or 25 [lane 5] μg/ml) for 45 min at 37°C prior to sonication and extraction of cell envelopes with CHAPS as described in Materials and Methods. Proteins were either run on SDS-polyacrylamide gels and stained with Coomassie brilliant blue (A) or electrophoretically transferred to nitrocellulose membranes and probed with MAbT11-30 (B). The positions of molecular mass markers (in kilodaltons) (right side) and the positions (left side) of the 57-kDa protein and the 38- and 29-kDa proteins which copurify with the 57-kDa protein after hemopexin affinity chromatography are indicated by the arrowheads.

were compared with known proteins in the Swiss-Prot database (University of Geneva, Geneva, Switzerland).

RESULTS

Hemopexin receptor affinity and abundance. Classical ligand-binding studies were carried out to define the characteristics of the hemopexin receptor on the surface of Hib strain 760705 grown in an iron-restricted medium. Hib 760705 bound heme–^{125}I-hemopexin in a time-dependent (Fig. 1) and saturable (displaceable) manner (Fig. 2), with saturation reaching a plateau after approximately 30 min at 37°C. The specificity of hemopexin binding was confirmed by the method of De Blasi et al. (5) (Fig. 2). Each ligand binds to a single class of binding site (Hill coefficients of 0.98 and 0.92 for heme-hemopexin and apohemopexin, respectively) and the affinities (K_ds) and receptor numbers were estimated to be 205 nM and 3,200 receptors per cell for heme-hemopexin and 392 nM and 4,400 receptors per cell for apohemopexin.

Surface exposure and contribution of the 57-kDa protein to hemopexin binding. Trypsin treatment of whole Hib cells has previously been shown to abolish hemopexin binding (43). We therefore sought to establish which of the three proteins of 57, 38, and 29 kDa isolated from detergent-solubilized membranes by hemopexin affinity chromatography may be responsible for hemopexin recognition. After trypsinization, the CHAPS-soluble fraction containing the hemopexin-binding proteins was subjected to SDS-PAGE and immunoblotting. Although progressive cleavage of the 57-kDa protein occurred, the 38- and 29-kDa proteins appeared relatively unaffected (Fig. 3). The 57-kDa protein appears to lose a small peptide fragment, leading to the appearance of a 52-kDa species on the SDS-polyacrylamide gel. This 52-kDa protein does not bind MAbT11-30 (Fig. 3A) or hemopexin (data not shown). Figure 3A also shows that the 30-kDa protein also recognized by MAbT11-30 (43), which comigrates with the 29-kDa protein in the gel shown, is also sensitive to trypsin.

Conservation of the 57- and 100-kDa hemopexin-binding proteins. To assess the conservation of hemopexin binding,

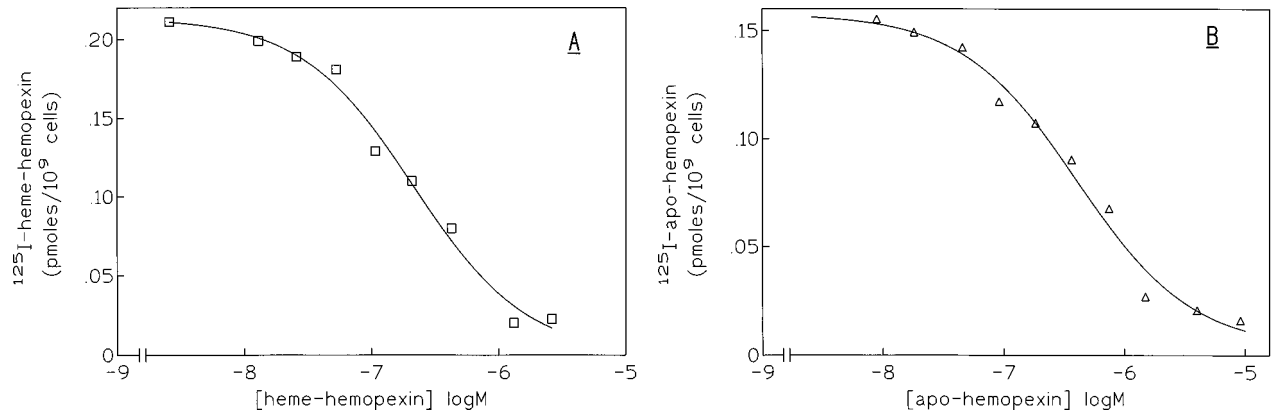

FIG. 2. Competition binding assay with human heme-hemopexin (A) or apohemopexin (B) as the radioligand and competitor. For these experiments, Hib strain 760705 was incubated for 30 min at 37°C with either 8.5 nM apo–^{125}I-hemopexin or heme–^{125}I-hemopexin in the presence of a range of concentrations of unlabelled human hemopexin (0 to 3 μM). Results depict the means and standard deviations of three experiments (the standard errors of the means were all smaller than the symbol size). From the curves obtained which were fitted with the curve-fitting program Graph Pad Inplot (ISI Inc.), the receptor numbers and K_ds were calculated as described by DeBlasi et al. (5).

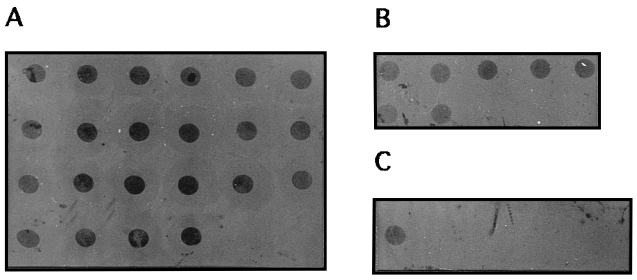

FIG. 4. Dot enzyme assay showing the binding of biotinylated human heme-hemopexin to whole cells of Hib (21 strains belonging to different outer membrane subtypes) (A), nontypeable *H. influenzae* (6 strains) (B), and *H. parainfluenzae* (5 strains) (C) immobilized on nitrocellulose membranes. Bacteria were grown in iron-restricted sBHI containing EDDA (100 μM). For each panel, the first dot (top left) is Hib strain 760705 (outer membrane subtype 1L).

whole-cell dot blots of Hib (21 strains belonging to different outer membrane subtypes), nontypeable *H. influenzae* (six strains) and *H. parainfluenzae* (five strains) grown in iron-restricted media were probed with biotinylated human hemopexin. All of the *H. influenzae* strains examined bound hemopexin, while *H. parainfluenzae* did not (Fig. 4).

Since the binding of hemopexin is presumably mediated through either the 57-kDa complex previously identified (43) or via the 100-kDa HxuA protein which may be surface associated (3) and since Hib strain 760705 lacks HxuA, we wished to investigate whether one or both of these hemopexin-binding proteins were conserved in *Haemophilus* strains. Outer membranes were prepared from each of the *Haemophilus* strains, and the outer membrane proteins were resolved by SDS-PAGE, blotted, and probed with MAbT11-30. All of the Hib (Fig. 5A) and nontypeable (Fig. 5B) strains contained a 57-kDa protein recognized by the monoclonal antibody. The *H. parainfluenzae* strains examined (Fig. 5C) lacked this protein, consistent with their inability to bind hemopexin.

We next determined whether the soluble 100-kDa HxuA protein is conserved among these *Haemophilus* strains. Ligand blots of the concentrated supernatants prepared from each strain were probed with biotinylated hemopexin (Fig. 6). Apart from strain 760705, all of the Hib and nontypeable *H. influenzae* strains produced the 100-kDa hemopexin-binding protein; none of the *H. parainfluenzae* strains examined produced this protein.

Identification of the 38-kDa protein as the porin P2. Since hemopexin binding in Hib strain 760705 correlates with the surface exposure of the 57-kDa protein as shown above, we wished to identify the 38- and 29-kDa proteins isolated by hemopexin affinity chromatography. Since the 38-kDa protein was resistant to trypsin (Fig. 3), a known characteristic of the 38-kDa P2 outer membrane porin protein of *H. influenzae* (37), we probed an immunoblot of the affinity-purified proteins with a monospecific antiserum raised against P2. The results shown in Fig. 7 indicate that the 38-kDa protein is P2. This hypothesis was confirmed by the results of NH_2-terminal amino acid sequence analysis which indicated that the first 10 amino acids of the 38-kDa protein (AVVYNNEGTN) were 100% identical to those described for the Hib outer membrane porin protein P2 (18). No sequence data could be obtained for the 29-kDa protein, which suggests that the NH_2 terminus is blocked.

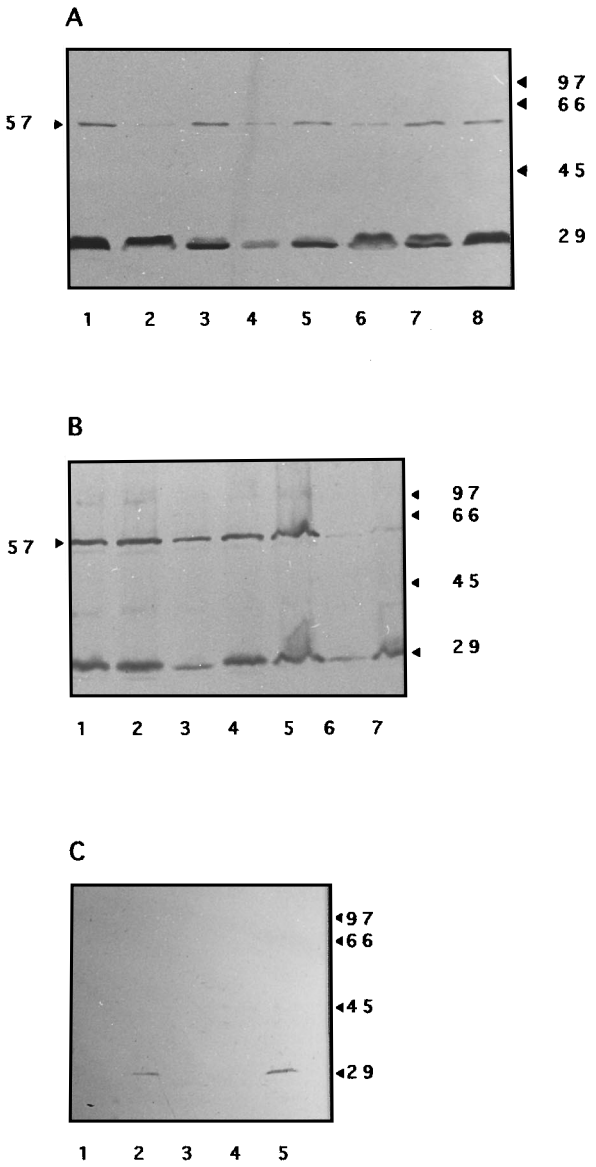

FIG. 5. Immunoblots of the outer membrane proteins of *H. influenzae* and *H. parainfluenzae* probed with MAbT11-30. (A) Hib strains belonging to outer membrane subtypes 3L (lane 1), 8H (lane 2), 9L (lane 3), 11L (lane 4), 15H (lane 5), 17L (lane 6), 19L (lane 7), and 1L (760705; lane 8). (B) Hib 760705 (lane 1) and nontypeable *H. influenzae* strains 2444 (lane 2), 2580 (lane 3), 2584 (lane 4), 2612 (lane 5), 2753 (lane 6), and 2844 (lane 7). (C) *H. parainfluenzae* strains P205 (lane 1), 8PS (lane 2), 16U (lane 4), 21E (lane 5), and DTL (lane 6). The arrowheads mark the positions of the 57-kDa hemopexin-binding protein (left-hand side) and the molecular mass standards (in kilodaltons) (right-hand side).

DISCUSSION

The multiplication of *H. influenzae* in host extracellular fluids is dependent in part on the ability of this human pathogen to compete with host proteins for essential nutrients such as iron and heme. Since these ligands are not normally freely available but are bound tightly to plasma transferrin and hemopexin (6, 23, 41), *H. influenzae* must have evolved mechanisms which facilitate the release and uptake of these protein-bound nutrients. For heme acquisition from hemopexin, *H. influenzae* binds hemopexin in a manner which meets the prerequisites for a biological receptor, namely, concentration-dependent binding which is saturable and ligand specific. Thus, *H. influenzae* expresses a receptor analogous to that described for hemopexin in a variety of cultured mammalian cells and tissues. However, this bacterial receptor exhibits a much lower

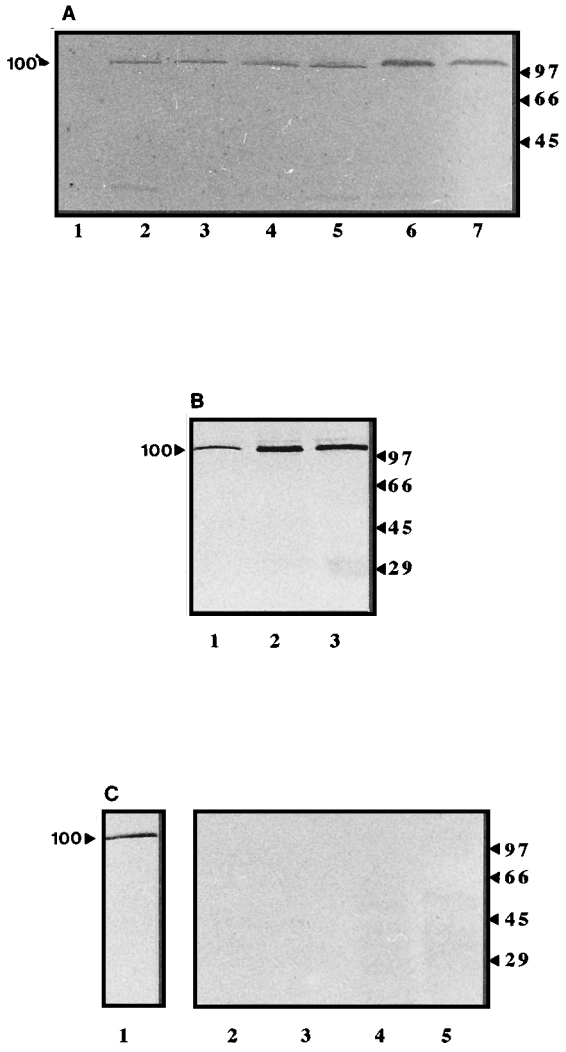

FIG. 6. Western blots of the proteins in cell-free supernatants of *H. influenzae* and *H. parainfluenzae* probed with biotinylated hemopexin. (A) Hib strains belonging to outer membrane subtypes 1L (760705) (lane 1), 1H (lane 2), 2L (lane 3), 3L (lane 4), 4H (lane 5), and 5L (lane 6), and Hib strain DL42 (lane 7). (B) Nontypeable *H. influenzae* strains 2444 (lane 1), 2580 (lane 2), and 2584 (lane 3). (C) Hib strain DL42 (lane 1) and *H. parainfluenzae* strains P205 (lane 2), 8PS (lane 3), 16U (lane 4), 21E (lane 5), and DTL (lane 6). The arrowheads mark the positions of the 100-kDa hemopexin-binding protein (left-hand side) and the molecular mass standards (in kilodaltons) (right-hand side).

affinity (K_d, 233 nM for heme-hemopexin) than that for human promyelocytic HL60 (K_d, 1 nM) (32), erythroleukemic K562 (K_d, 5 nM) (31), or mouse hepatic cells (K_d, 15 nM) (25) or for the human placenta (K_d, 66 nM) (30). Consistent with these observations for the hemopexin receptor, the binding of transferrins to prokaryotic transferrin receptors is also of relatively low affinity with K_ds in the low micromolar range in contrast to the nanomolar affinities of the mammalian receptor for iron-saturated transferrin. For example, the diferric transferrin receptors of *Neisseria meningitidis* (33) and *Staphylococcus aureus* (13) have K_ds of 0.7 and 0.28 μM, respectively, compared with the rabbit reticulocyte diferric transferrin receptor (K_d, 11 nM) (47). At present, it is not at all clear why these bacterial plasma protein receptors have such comparatively low affinities for their respective ligands.

In contrast to the mammalian hemopexin receptor, *H. influenzae* exhibits only an approximately twofold-greater affinity for the heme-loaded ligand. Such a difference may ultimately provide important insights into the nature of the receptor-ligand interaction in bacteria which do not endocytose receptor-bound proteins. Upon binding heme, hemopexin undergoes a major conformational change in which the heme-binding N-terminal lobe of the protein (domain I) assumes a much more compact shape with an enhanced interaction between the two domains, I and II (14, 23, 45, 46). This conformational change appears to play a primary role in recognition by the mammalian receptor (14, 27), and a receptor-binding site has recently been located on the C-terminal side of the hemopexin heme-binding histidine residue 127 (14, 45, 46). Since the affinities of binding of apo- and heme-hemopexin by *H. influenzae* are similar, it is possible that this human pathogen interacts with regions of hemopexin which differ from the mammalian receptor-binding sites and which may be unaffected by heme binding.

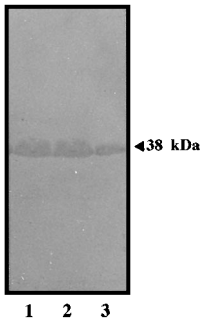

FIG. 7. Immunoblot of the whole-cell envelope proteins (lane 1). Triton X-100-extracted outer membrane proteins (lane 2) and hemopexin-Sepharose 4B affinity-isolated hemopexin-binding proteins (lane 3) of Hib 760705 probed with an antiserum raised against outer membrane porin protein P2. The arrowhead indicates the position of the 38-kDa P2 protein.

Hemopexin affinity chromatography enables the isolation of three proteins of 57, 38, and 29 kDa from Hib (43). Since protease treatment of whole cells abolishes hemopexin binding, we sought to determine whether any of these three proteins is degraded. Loss of hemopexin binding correlated with cleavage of only the 57-kDa protein. Since this protein binds hemopexin in ligand blot assays, it appears to be the major surface-exposed component of the outer membrane hemopexin receptor. Furthermore, trypsin appears to cleave an approximately 5-kDa peptide fragment from the 57-kDa hemopexin-binding protein, generating a 52-kDa species which does not bind hemopexin. This result implies that the 5-kDa fragment must have been derived from either the N terminus or the C terminus of the 57-kDa protein and that this fragment either contains or is part of the hemopexin-binding site. Interestingly, the epitope recognized by MAbT11-30 lies close to this region, since the 52-kDa cleavage product does not bind the monoclonal antibody.

The data derived from the ^{125}I-hemopexin-binding studies which provide evidence for a single surface hemopexin-binding site together with the trypsinization experiments suggest that the 57-kDa protein provides the principal surface recognition of hemopexin. This raises the possibility that the 29- and 38-kDa proteins isolated as a complex with the 57-kDa protein are accessory proteins which may function in the release of heme from hemopexin and its subsequent transport into the cell. For liver parenchymal cells, two receptor-mediated processes for the uptake of heme from hemopexin have been described elsewhere (23, 24). The heme-hemopexin complex is endocy-

tosed in a manner analogous to that of diferric transferrin. Heme is then released from hemopexin intracellularly and apohemopexin is recycled intact to the exterior (24). In addition, there is another process which involves the receptor functioning in an enzyme-like manner and catalyzing the transfer of heme from hemopexin to a membrane heme-binding protein, possibly prior to translocation into the cell (26). Since bacteria do not endocytose proteins, it is likely that *H. influenzae* releases heme from hemopexin at the bacterial cell surface. Indeed, *H. influenzae* has been reported to transport the intact heme molecule across the cell envelope by an active process (4) recently shown to be dependent on a functional *tonB* gene (9) and to possess an iron-regulated outer membrane heme-binding protein of 39.5 kDa (11).

In an attempt to identify the 38- and 29-kDa proteins, N-terminal amino acid sequencing was performed. Although we were unable to obtain any data for the 29-kDa protein, the antibody cross-reactivity data together with the amino acid sequence clearly indicate that the 38-kDa protein is the porin protein P2. Since P2 copurifies with the 57- and 29-kDa proteins, it is tempting to speculate on its possible contribution to hemopexin binding and heme internalization. P2 is a constitutively expressed and abundant outer membrane protein (18, 37). Since hemopexin binding in Hib strain 760705 is regulated by iron (43), P2 may contribute to the receptor complex by promoting the correct organization and arrangement of the 57- and 29-kDa proteins in the outer membrane so that heme from heme-hemopexin can readily be released and internalized. It is also conceivable that such an arrangement may facilitate the internalization of heme released from hemopexin through the P2 porin, since it has a pore size of around 1,400 Da, large enough to admit passage of hemin molecules (652 Da) (35). However, heme is a hydrophobic molecule and therefore is unlikely to diffuse through the aqueous transmembrane channels formed by the P2 porin protein. As yet, no evidence has been presented in the literature to indicate that the permeability of the *H. influenzae* cell envelope to heme is due to the presence of P2. It is perhaps worth noting that the outer membranes of *Escherichia coli* K-12 and *Salmonella typhimurium* which are impermeable to heme (12, 22) contain porins which have exclusion limits of around 600 Da (19). Clearly, further work will be required to define the contribution of P2 to the uptake of heme from hemopexin.

Heme uptake from hemopexin by *H. influenzae* has been reported by others to be dependent on a soluble secreted 100-kDa hemopexin-binding protein termed HxuA (3, 7). Although Hib strain 760705 does not express HxuA, it nevertheless possesses the *hxuA* gene (3). Furthermore, 760705 can utilize heme from hemopexin after growth in an iron-restricted, porphyrin-sufficient medium (43). In addition, using colony blot assays, Hanson et al. (7) and Cope et al. (3) were unable to demonstrate the binding of hemopexin to nontypeable *H. influenzae* strains even though they could utilize heme from heme-hemopexin and express the 100-kDa hemopexin-binding protein. To resolve these apparent discrepancies, we first sought to determine the relative conservation of hemopexin binding and expression of both the 57- and 100-kDa proteins. This approach was carried out with a collection of Hib strains belonging to different outer membrane subtypes as well as with a group of nontypeable strains and *H. parainfluenzae*. After growth in an iron-restricted medium, all of the Hib and nontypeable strains examined bound hemopexin on dot blots. This is therefore the first time that whole cells of nontypeable *H. influenzae* strains have been shown to bind hemopexin. Furthermore, both the 57- and 100-kDa hemopexin-binding proteins were found to be highly conserved in *H. influenzae*; only Hib strain 760705 lacks this 100-kDa protein. In contrast, none of the *H. parainfluenzae* strains examined bound hemopexin or possessed either the 57- or the 100-kDa hemopexin-binding protein. This is not perhaps surprising, given that *H. parainfluenzae* does not have an essential requirement for heme, although it can use heme and hemoglobin as an iron source (42). It is also unable to sequester iron from transferrin or heme from hemoglobin-haptoglobin complexes (42). Thus, the multiple mechanisms found in *H. influenzae* for acquiring protein-bound iron and heme may provide this pathogen with the added capacity to cause invasive disease.

Although Hib strain 760705 does not express the soluble HxuA protein, it can both bind heme-hemopexin and utilize heme from hemopexin after growth in an iron-restricted medium. Thus, it appears that the 100-kDa protein is not essential for this process. Furthermore, the nontypeable strain OC201, which expresses a functional HxuA protein, is unable to bind or utilize heme from hemopexin for growth (3). The question, then, is why do most *H. influenzae* strains express both the 57-kDa outer membrane protein and the secreted 100-kDa hemopexin-binding protein. Recently, the uptake of heme from hemopexin in *H. influenzae* has been shown to be dependent on a functional TonB protein (9). In *E. coli*, TonB functions as an energy transducer to promote the active transport of iron siderophores and vitamin B_{12} across the outer membrane (10, 21). TonB is anchored to the periplasmic face of the cytoplasmic membrane and appears to physically interact with TonB-dependent outer membrane proteins, such as the enterobactin receptor protein FepA, through a conserved peptide known as the TonB box (10, 21). HxuA is a soluble secreted protein which does not contain any of the motifs characteristic of TonB-dependent outer membrane proteins (3). Since Hib strain 760705 does not express HxuA, uptake of heme from hemopexin in *H. influenzae* strains expressing the soluble 100-kDa HxuA protein must involve outer membrane components. Expression of most of the established TonB-dependent outer membrane proteins is known to be regulated by iron (10, 21), and we have shown that hemopexin binding is dependent on the availability of iron in the growth medium (43). It is therefore possible that the 57-kDa hemopexin-binding protein complex may constitute a TonB-dependent outer membrane heme transport system. Further work is under way to clone and sequence the 57-kDa hemopexin-binding protein and to elucidate the nature of its contribution to the uptake of heme from hemopexin.

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