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Binding and Accumulation of Hemin in *Neisseria gonorrhoeae*

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The ability to utilize hemin and hemin-containing compounds for nutritional iron (Fe) uptake has been documented for several pathogenic bacteria. *Neisseria gonorrhoeae* can utilize free hemin as a source of Fe for growth; however, little is known concerning the mechanisms involved in hemin transport. In this study we have characterized the binding and accumulation of hemin by *N. gonorrhoeae* and defined the specificity of the gonococcal hemin receptor. *N. gonorrhoeae* F62 was grown in a chemically defined medium containing the iron chelator Desferal, and hemin transport was initiated by the addition of [^{59}Fe]hemin (4.0 or 8.0 μM; specific activity, 7.0 Ci/mol). ^{59}Fe uptake from radiolabeled hemin by *N. gonorrhoeae* was energy dependent, and ^{59}Fe was shown to accumulate in the cell at a constant rate during logarithmic growth. However, we observed a decrease in the uptake of ^{59}Fe from radiolabeled hemin when inorganic iron was present in the growth medium. Binding of ^{59}Fe from radiolabeled hemin was inhibited by the addition of either cold hemin, hematoporphyrin, or hemoglobin, but not by ferric citrate. Although [^{14}C]hemin was found to support the growth of *N. gonorrhoeae*, we did not detect the uptake of ^{14}C from radiolabeled hemin. Extraction of the gonococcal periplasmic ferric binding protein (Fbp) from cultures grown with [^{59}Fe]hemin indicated that a majority of the ^{59}Fe was associated with the Fbp. Taken together, the results presented here indicate that hemin binds to a gonococcal outer membrane receptor through the protoporphyrin portion of the molecule and that following binding, iron is removed and transported into the cell, where it is associated with the gonococcal periplasmic ferric binding protein, Fbp.

The low concentration of free iron in body fluids creates bacteriostatic conditions for many microorganisms and is an important defense factor against invading bacteria. Consequently, pathogenic organisms have developed diverse and elaborate systems to obtain the iron required for growth (61). Many microorganisms synthesize high-affinity iron chelators (siderophores) as well as specific transport systems for the transport of siderophore-Fe^{3+} complexes into the cell. Some organisms can acquire iron directly from host iron-binding proteins by highly specific recognition and transport systems. Within the human host potential iron sources available to pathogenic bacteria include transferrin (TF) found in serum and lactoferrin (LF) present on mucosal surfaces (61). In addition, heme-containing compounds are a particularly abundant source of in vivo iron. Pathogens that occupy intracellular niches in vivo can utilize heme directly. However, extracellular pathogenic bacteria can utilize the iron in heme compounds only after the heme is released; this typically occurs by some form of tissue damage resulting in the release of intracellular material (48). The ability to utilize hemin and hemin-containing compounds for nutritional iron uptake has been documented for several pathogenic bacteria, including *Vibrio cholerae*, *Vibrio vulnificus*, *Klebsiella pneumoniae*, *Yersinia pestis*, *Yersinia enterocolitica*, *Shigella flexneri*, *Bacteroides fragilis*, *Bordetella pertussis*, *Streptococcus pneumoniae*, *Haemophilus ducreyi*, *Haemophilus influenzae*, *Serratia marcescens*, *Plesiomonas shigelloides*, and *Porphyromonas gingivalis* (12, 18, 22, 28, 31, 38, 40, 48, 49, 54).

The gram-negative pathogen *Neisseria gonorrhoeae* is capable of utilizing a wide variety of iron sources, including TF, LF, and heme-containing compounds (13, 18, 41–43). Although hemoglobin bound to haptoglobin can be used as an iron source by most gonococcal strains, heme cannot be used when it is complexed with hemopexin or albumin (18). *N. gonorrhoeae* does not produce siderophores, and acquisition of iron from TF and LF involves a family of distinct iron-regulated outer membrane receptors (2, 11, 16, 60). These include the well-characterized TF-binding proteins, Tbp1 and Tbp2, and the LF-binding protein, Lbp (2, 10, 37, 59). Iron is removed from TF and LF in an energy-dependent manner; however, neither TF nor LF is internalized. Once the iron from TF has been internalized, it appears to be transported onto the ferric binding protein (Fbp) (46, 47). Fbp appears to function within the periplasm, shuttling iron from TF through the periplasm and to the cytoplasmic membrane (5, 47). The gene for the gonococcal and meningococcal Fbp has been cloned and sequenced (6, 7), and the promoter region has been shown to contain two regions that exhibit homology with the *Escherichia coli* ferric uptake regulator protein (Fur) binding sequence (4, 7). Fbp appears to be regulated by a Fur-like mechanism, and gonococcal and meningococcal Fur homologs have been identified (8, 32, 56).

Although the ability of *N. gonorrhoeae* to utilize hemin and hemin-containing compounds has been documented, the mechanisms whereby this organism acquires hemin are poorly understood. Two hemin-binding proteins (97 and 44 kDa) have been purified from *N. gonorrhoeae* by hemin-affinity chromatography (34); however, the role of these proteins in hemin utilization has not been established. A haptoglobin-hemoglobin-binding protein and a hemoglobin binding receptor (HmbR) have been described for *Neisseria meningitidis* (39, 55); however, it is not known if similar homologs exist in *N. gonorrhoeae*. The ability to acquire heme iron may be particularly important for a mucosal pathogen like *N. gonorrhoeae*, since a large amount of hemin is present at mucosal sites because of the desquamation of epithelial cells. In this study, we have characterized the transport of hemin by *N. gonor-*

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rhoeae, defined the specificity of the hemin receptor, and determined the role of Fbp in hemin transport.

MATERIALS AND METHODS

Bacteria and growth conditions. *N. gonorrhoeae* F62 (obtained from R. P. Williams, Baylor College of Medicine, Houston, Tex.) was typically maintained on gonococcal base (GCB) medium containing 1% Kellogg's supplement (21) and grown aerobically (5% CO_2) at 37°C. Broth cultures were grown in chemically defined medium (CDM) supplemented with 4.2% $NaHCO_3$ as previously described (21). For hemin transport studies, *N. gonorrhoeae* was grown in CDM containing the iron chelator Desferal (25.0 μM; Ciba-Geigy) (CDM/25D) for 3 h. This culture served as the inoculum for hemin transport studies as described below. Growth was monitored spectrophotometrically at 660 nm, and viability of cultures was assessed by serial dilution and plating on GCB followed by incubation for 24 h as described above.

Hemin transport studies. All glassware was washed with 10% nitric acid and thoroughly rinsed in deionized water to remove residual iron. [^{59}Fe]hemin (specific activity, 7.0 Ci/mol) was purchased from Dupont, NEN Research Products (Boston, Mass.). [^{14}C]hemin was purchased from University of Leeds, Innovations, Ltd., Leeds, England; cold hemin (Na plus K salt) and hematoporphyrin were purchased from Porphyrin Products Inc. (Salt Lake City, Utah); and apotransferrin and hemoglobin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Radiolabeled hemin was dissolved in 0.1 N NaOH, aliquoted, and stored at −80°C until use. Cold hemin was dissolved in distilled H_2O and prepared fresh for each experiment.

N. gonorrhoeae F62 was grown in CDM/25D for 3 h to limit the Fe content, and cells were washed, resuspended in fresh CDM containing 12.5 μM Desferal (CDM/12.5D) or in CDM/12.5D containing 100 to 200 μM KCN, and incubated for 30 min at 37°C. Hemin transport was initiated by the addition of [^{59}Fe]hemin (4.0 μM; specific activity, 7.0 Ci/mol) or [^{14}C]hemin (0.125 μM; specific activity, 105 Ci/mol) supplemented with 7.875 μM cold hemin, and cultures were incubated at 37°C. Duplicate 1.0-ml samples were removed at hourly intervals, diluted into 10 ml of 0.1 M sodium citrate (pH 7.4) containing 1 μM $MgCl_2$ and 0.25 mM $CaCl_2$, and filtered through 0.45-μm-pore-size cellulose acetate filters. The filters were then washed three times with 1.0 ml of 0.02 N NaOH in 50% ethanol and air dried overnight, and the amount of cell-associated ^{14}C or ^{59}Fe from radiolabeled hemin was determined by liquid scintillation spectrometry. Cell-associated ^{59}Fe was expressed as picomoles per milligram of total cellular protein; hemin uptake was calculated as the difference between the amount of ^{59}Fe from radiolabeled hemin associated with non-KCN-treated cultures and the amount from radiolabeled hemin associated with KCN-treated cultures and was expressed as picomoles per milligram of total cellular protein for each time point. The amount of cell-associated ^{14}C was determined from the counts per minute of non-KCN-treated cultures in samples containing equivalent amounts of protein. Total protein content was determined for each sample by use of the Bio-Rad (Hercules, Calif.) protein assay with bovine serum albumin as the standard.

Competition assays. For competition assays, *N. gonorrhoeae* F62 was grown in CDM/25D for 3 h to limit the Fe content, and cells were washed, resuspended in fresh CDM/12.5D or CDM/12.5D containing 100 to 200 μM KCN, and incubated for 30 min at 37°C. Hemin transport was initiated by the addition of [^{59}Fe]hemin (4.0 or 8.0 μM) and a competitor, i.e., unlabeled hemin (4 or 16 μM), hematoporphyrin (4 or 8 μM), apotransferrin (0.8 or 8.0 μM), or hemoglobin (0.8 or 8.0 μM). The uptake of radiolabeled hemin was determined by liquid scintillation spectrometry. Cell-associated ^{59}Fe from radiolabeled hemin was expressed as described above or as picomoles per 10^7 CFU for competition experiments using hemoglobin or transferrin. For each time point hemin uptake was calculated as the difference between the amount of [^{59}Fe]hemin associated with non-KCN-treated cultures and the amount associated with KCN-treated cultures and expressed as picomoles per 10^7 CFU (for hemoglobin and transferrin competition experiments) or picomoles per milligram of protein (for hemin, hematoporphyrin, and Fe-citrate competition experiments). Competition was assessed by comparing [^{59}Fe]hemin binding and uptake in *N. gonorrhoeae* cultures containing competitors with cultures that contained only [^{59}Fe]hemin. Data are expressed as percent uptake and percent binding and as percent inhibition of uptake and percent inhibition of binding.

Fbp extraction. For Fbp extraction studies, *N. gonorrhoeae* F62 was grown in CDM/25D to the mid-exponential phase of growth. This culture was used to inoculate 500 ml of prewarmed CDM/25D, and incubation was continued until the culture reached the early stationary phase of growth. Cells were harvested by centrifugation (12,000 × *g*) at 4°C, and the cell pellet was resuspended in fresh CDM/12.5D supplemented with [^{59}Fe]hemin (4.0 μM; specific activity, 7.0 Ci/mol) in the presence or absence of 100 μM KCN. After 2 h of incubation at 37°C, cells were harvested by centrifugation and washed in phosphate-buffered saline and the Fbp was extracted with cetyltrimethylammonium bromide (Sigma) and purified as described previously (45). After CM Sepharose (Sigma) chromatography, the radioactivity associated with each fraction was quantitated by liquid scintillation spectrometry and the protein profiles in the peak fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The presence of Fbp was confirmed by pooling protein-containing fractions and examining them by Western blot (immunoblot) analysis using rabbit anti-Fbp antisera (24).

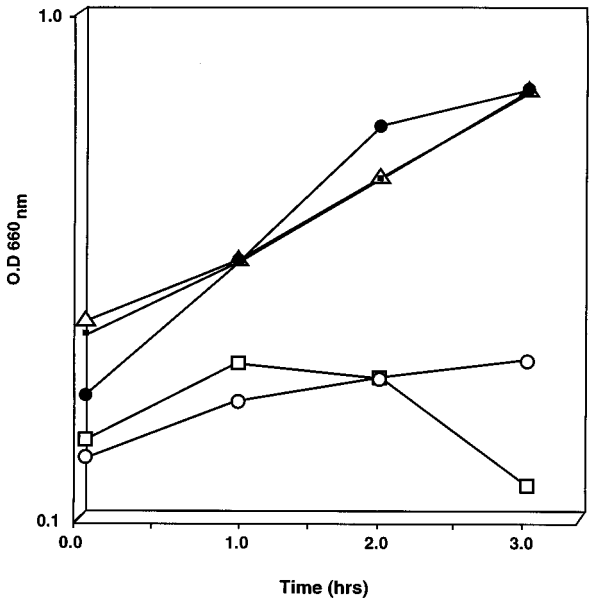

FIG. 1. Growth of *N. gonorrhoeae* F62 with [^{59}Fe]hemin and [^{14}C]hemin. Gonococci were grown in CDM/25D for 3 h, and cells were suspended in fresh CDM/12.5D (□) or CDM/12.5D supplemented with 0.5 μM [^{14}C]hemin (△), 4.0 μM [^{14}C]hemin (■), 4.0 μM [^{59}Fe]hemin (●), or 4.0 μM [^{59}Fe]hemin containing 100 μM KCN (○). Growth was monitored by the A_{660}. Results are from one experiment and are representative of three separate experiments. O.D 660_{nm}, optical density at 660 nm.

RESULTS

Effect of hemin and iron restriction on growth of *N. gonorrhoeae* F62. When *N. gonorrhoeae* F62 was grown in CDM without added iron, the culture continued to grow for several generations (data not shown). These cells apparently had accumulated residual iron present in CDM and utilized this endogenous pool until exhausted. However, when *N. gonorrhoeae* was initially grown in CDM for 3 h and then reinoculated into fresh CDM containing Desferal (12.5 μM), minimal growth was observed (Fig. 1). Therefore, for all assays described below, *N. gonorrhoeae* was grown in CDM/25D and this culture served as the inoculum for radiolabeled hemin studies. Growth of *N. gonorrhoeae* F62 in CDM/12.5D supplemented with [^{59}Fe]hemin (4.0 μM) or [^{14}C]hemin (4.0 μM) resulted in a typical growth curve with a final cell density of 10^{10} CFU (Fig. 1 and data not shown). *N. gonorrhoeae* grown in CDM/12.5D supplemented with lower concentrations of [^{14}C]hemin (0.5 μM) exhibited a typical growth curve, although the final cell density of 10^8 CFU/ml was lower than that of cultures grown in CDM supplemented with 4.0 μM hemin (Fig. 1 and data not shown).

Hemin binding and accumulation by *N. gonorrhoeae* as assessed with [^{59}Fe]hemin. To examine the kinetics of hemin binding under growing conditions, we determined *N. gonorrhoeae* hemin binding with [^{59}Fe]hemin as the sole iron source during growth in CDM/12.5D. For these studies, cultures of *N. gonorrhoeae* F62 were grown under iron-depleted conditions (CDM/25D) for 3 h and reinoculated into fresh CDM/12.5D. Binding was determined under growing conditions by supplementation of cultures with [^{59}Fe]hemin (4.0 μM), and hemin binding was expressed as the total amount of hemin associated

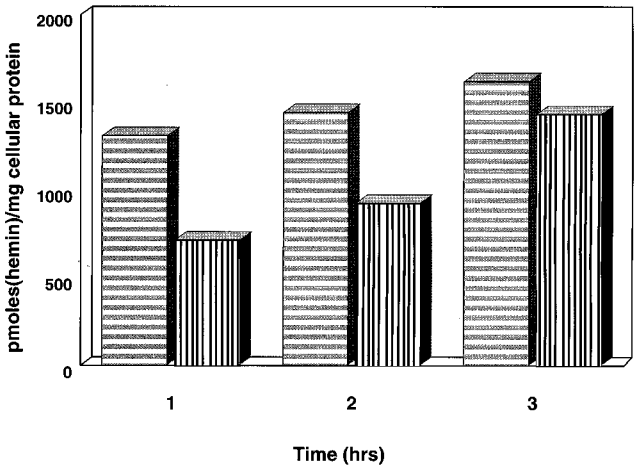

FIG. 2. [^{59}Fe]hemin binding and transport by *N. gonorrhoeae* F62. *N. gonorrhoeae* F62 was grown in CDM/25D for 3 h, and cells were suspended in fresh CDM/12.5D supplemented with [^{59}Fe] hemin (4.0 μM; specific activity, 7.0 Ci/mol). Hemin binding and transport were determined under growing conditions with radiolabeled hemin as a function of time as described in Materials and Methods. Levels of [^{59}Fe]hemin binding (▤) and uptake (▥) are shown. The amount of hemin bound was determined from the total hemin associated with whole cells and is expressed as picomoles per milligram of total cellular protein for each time point. Hemin uptake was calculated as the difference between the amount of [^{59}Fe]hemin associated with untreated cultures and the amount associated with KCN-treated cultures and is expressed as picomoles per milligram of total cellular protein for each time point. Results are from one experiment and are representative of three separate experiments.

with whole cells. We did not observe precipitation or filter binding of [^{59}Fe]hemin in uninoculated media (data not shown). Hemin binding was initially detected at 1 h, and the amount of hemin bound by *N. gonorrhoeae* increased over time, with maximal binding by 3 h (Fig. 2). *N. gonorrhoeae* grown in CDM/12.5D bound 1,614 pmol/mg of cellular protein, as detected at 3 h.

To confirm the active transport of hemin, KCN, an inhibitor of energy transduction (17), was used. The inhibition of growth by KCN was confirmed by optical density readings and viable cell counts (Fig. 1 and data not shown). The accumulation of hemin was calculated as the difference between the amount of ^{59}Fe from radiolabeled hemin associated with non-KCN-treated cells (total hemin) and the amount from radiolabeled hemin associated with KCN-treated cells and was expressed as picomoles per milligram of total cellular protein for each time point. As shown in Fig. 2, ^{59}Fe from radiolabeled hemin was transported by *N. gonorrhoeae* in an energy-dependent fashion and accumulated in the cell at a constant rate during logarithmic growth. Cultures of *N. gonorrhoeae* grown in CDM/12.5D and sampled at 1 h accumulated 713 pmol of hemin per mg of protein (Fig. 2). This time point is equivalent to approximately one generation, as determined by the optical density and number of CFU (Fig. 1 and data not shown). Cultures exhibited typical growth as determined by viable counts and protein concentration, indicating that radiolabeled hemin was sufficient to support bacterial growth (Fig. 1 and data not shown). Accumulation of hemin by *N. gonorrhoeae* continued steadily; by 3 h *N. gonorrhoeae* accumulated 1,431 pmol of hemin per mg of protein (Fig. 2). The increased accumulation seen over the 3-h period was due to the uptake and incorporation of ^{59}Fe from radiolabeled hemin into the cell.

Binding and accumulation of [^{14}C]hemin by *N. gonorrhoeae*. In *P. gingivalis*, *H. influenzae*, *Y. enterocolitica*, *P. shigelloides*, and *V. cholerae*, the entire hemin molecule is transported into the cell (18, 20, 26, 27, 29, 54). The results obtained here with [^{59}Fe]hemin indicated that *N. gonorrhoeae* can transport the Fe from hemin into the cell. To determine if *N. gonorrhoeae* can transport the protoporphyrin (PPIX) ring into the cell, we examined hemin accumulation with [^{14}C]hemin. As shown in Table 1, we detected binding of ^{14}C from radiolabeled hemin to the gonococcal cell surface; this is represented as non-KCN-treated cells. However, when we calculated hemin uptake, i.e., the difference between the amount of ^{14}C from radiolabeled hemin associated with non-KCN-treated cultures and the amount from radiolabeled hemin associated with KCN-treated cultures (Table 1), we found that ^{14}C from radiolabeled hemin did not accumulate in the cell. [^{14}C]hemin was found to support the growth of *N. gonorrhoeae* (Fig. 1), indicating that sufficient iron could be obtained from [^{14}C]hemin. Taken together, these results suggest that *N. gonorrhoeae* can remove the iron from the hemin molecule prior to internalization and transport of inorganic iron into the cell. Although the PPIX ring does not appear to be taken into the cell, it does appear to be involved in the binding of hemin to the gonococcal cell surface.

Competition for hemin binding and accumulation by hematoporphyrin or Fe-citrate. To determine if hemin, hematoporphyrin, or Fe-citrate could compete with radiolabeled hemin for binding and uptake, and to confirm the specific accumulation of hemin, we examined the uptake of radiolabeled hemin in the presence of cold hemin, hematoporphyrin, or Fe-citrate. Both hematoporphyrin and hemin were found to compete for hemin binding (Table 2). Cultures of *N. gonorrhoeae* to which 16.0 μM cold hemin had been added bound 71% of the hemin bound by control cultures, a 29% reduction in hemin bound (Table 1). Likewise, hemin accumulation by these cultures was inhibited by 34%.

The addition of hematoporphyrin resulted in a 43% inhibition of hemin binding and a 55% inhibition of hemin accumulation as detected at 1 h (Table 2). Interestingly, although Fe-citrate was found to inhibit hemin accumulation by *N. gonorrhoeae* (54% inhibition of uptake), we did not observe inhibition of binding (Table 2). In addition, we observed a decrease in the uptake of ^{59}Fe from radiolabeled hemin by *N. gonorrhoeae* when inorganic iron was present in the growth medium (data not shown). These results suggest that the uptake of Fe from hemin may not be as efficient a system for iron uptake as the system used for the uptake of inorganic iron. Reduced accumulation of hemin by cells grown with inorganic iron also indicates that the *N. gonorrhoeae* hemin transport system is regulated by iron and probably by Fur. In addition,

TABLE 1. [^{14}C]hemin binding and accumulation by *N. gonorrhoeae*[a]

Time (h)	[^{14}C]hemin binding and accumulation (cpm)	
	NKCN	KCN
1	924	1,921
2	937	882
3	957	702
4	1,257	1,001

[a] *N. gonorrhoeae* F62 was grown in CDM/25-D for 3 h, and cells were suspended in fresh CDM/12.5D supplemented with [^{14}C]hemin (0.125 μM; specific activity, 105 Ci/mol) and 7.875 μM cold hemin. Levels of hemin binding and accumulation were determined under growing conditions as a function of time. The amount of hemin bound was determined from the total hemin associated with whole cells (non-KCN-treated cultures [NKCN]) and is expressed as total counts per minute for each time point. Results are from one experiment and are representative of five separate experiments. KCN, KCN-treated cultures.

TABLE 2. [^{59}Fe]hemin binding and transport by *N. gonorrhoeae* in the presence of hemin, hematoporphyrin, Fe-citrate, hemoglobin, or TF[a]

Competitor[b]	% [^{59}Fe]hemin binding[c]	% Inhibition of binding	% [^{59}Fe]hemin uptake[c]	% Inhibition of uptake
Hemin[d]				
4 μM (1×)	96	4	80	20
16 μM (4×)	71	29	66	34
Hematoporphyrin, 4 μM (1×)[d]	57	43	45	55
Fe-citrate, 4 μM (1×)[d]	100	0	46	54
Hemoglobin[e]				
4 μM (1×)	1	99	0	100
8 μM (1×)	4	96	0	100
TF[e]				
0.8 μM (0.1×)	93	7	100	0
8.0 μM (1×)	49	51	46	54

[a] Levels of hemin binding and transport were determined under growing conditions with [^{59}Fe]hemin (4.0 or 8.0 μM; specific activity, 7.0 Ci/mol).
[b] Numbers in parentheses refer to the amount of competitor added relative to radiolabeled hemin.
[c] Percents [^{59}Fe]hemin binding and uptake were determined relative to controls that did not contain a competitor. Results are from one experiment and are representative of three separate experiments.
[d] For hemin, hematoporphyrin, and Fe-citrate competition experiments the amount of hemin bound was determined from the total hemin associated with whole cells and expressed as picomoles per milligram of total cellular protein detected at 1 h. Hemin uptake was calculated as the difference between the amount of [^{59}Fe]hemin associated with untreated cultures and the amount associated with KCN-treated cultures and expressed as picomoles per milligram of total cellular protein.
[e] For hemoglobin and TF competition experiments, the amount of hemin bound was determined from the total hemin associated with whole cells and expressed as picomoles per 10^7 cells as detected at 2 h. Hemin uptake was calculated as the difference between the amount of [^{59}Fe]hemin associated with untreated cultures and the amount associated with KCN-treated cultures and expressed as picomoles per 10^7 cells.

these results indicate that a putative gonococcal hemin-binding protein specific for the PPIX ring is required for the binding and transport of hemin.

Specificity of the hemin binding receptor: competition for hemin binding and accumulation by hemoglobin or transferrin. Acquisition of iron from TF, LF, hemoglobin, or hemoglobin-haptoglobin in pathogenic *Neisseria* spp. involves a family of distinct outer membrane receptors (16, 39, 55, 60). To determine if TF or hemoglobin could bind to the gonococcal cell surface and inhibit the binding of hemin, we examined the binding and accumulation of radiolabeled hemin in the presence of apotransferrin or hemoglobin. Addition of hemoglobin (0.8 μM) to *N. gonorrhoeae* cultures in CDM/12.5D containing 4.0 μM [^{59}Fe]hemin resulted in the inhibition of both hemin binding and hemin accumulation (data not shown). This inhibition was observed when hemoglobin was preincubated with gonococcal cultures for 1 h prior to the addition of [^{59}Fe]hemin and when hemoglobin and [^{59}Fe]hemin were added simultaneously (data not shown). Cultures of *N. gonorrhoeae* to which 8.0 μM hemoglobin had been added simultaneously with [^{59}Fe]hemin (8.0 μM) bound 4% of the hemin bound by control cultures, a 96% reduction in hemin bound, as detected at 2 h (Table 2). Hemin accumulation by these cultures was reduced by 100% (Table 2). The addition of apotransferrin (0.8 μM) resulted in only a 7% inhibition of hemin binding and did not inhibit hemin accumulation (Table 2). Addition of 8.0 μM apotransferrin resulted in 51% inhibition of hemin binding and 54% inhibition of hemin uptake.

Interestingly, we observed that the addition of hemoglobin (4.0 μM) or hematoporphyrin (4.0 μM) resulted in a greater inhibition of [^{59}Fe]hemin uptake than did the addition of cold hemin (4.0 μM) (Table 2). The observed reduced capacity of cold hemin to inhibit binding and uptake of [^{59}Fe]hemin compared with hemoglobin and hematoporphyrin may reflect the tendency of hemin to aggregate in aqueous solutions (36), resulting in a reduction of the concentration of available hemin. The inhibition observed with hemoglobin could be due to the use of a common receptor for the utilization of hemin from hemoglobin. Alternatively, hemin could be readily removed from the globin molecule and be available to interact directly with the putative gonococcal hemin-specific receptor.

Association of iron from hemin with Fbp. To examine the association of iron from hemin with the gonococcal Fbp, [^{59}Fe]hemin was added to iron-starved gonococcal cultures and the amount of ^{59}Fe associated with Fbp was determined. After a 2-h incubation, cells were harvested and washed and the Fbp was purified from cetyltrimethylammonium bromide extracts by cation-exchange chromatography. This protocol has previously been demonstrated to enrich for Fbp (45). The protein content as monitored by A_{280} and the amount of labeled iron were determined for each fraction, and the results are shown in Fig. 3. When the culture was grown with [^{59}Fe]hemin as the sole source of iron, the majority of the ^{59}Fe in the cetyltrimethylammonium bromide extract containing Fbp was found to be associated with the gonococcal Fbp. In the absence of KCN, Fbp eluted as a single peak and was associated with a

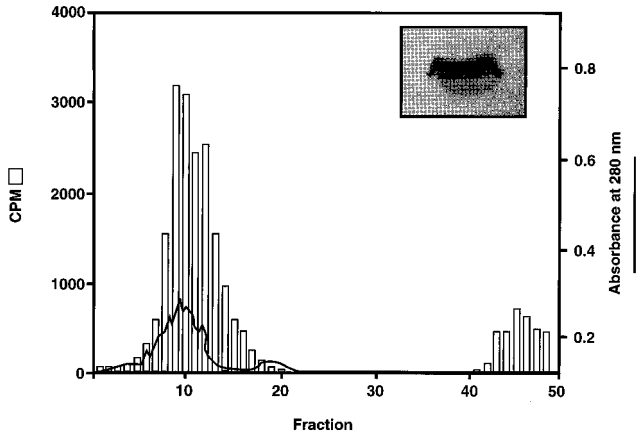

FIG. 3. Association of ^{59}Fe from hemin with Fbp. *N. gonorrhoeae* F62 was grown in CDM/25D for 3 h, and cells were suspended in fresh CDM/25D containing [^{59}Fe]hemin (4.0 μM). After 2 h of incubation, cells were harvested and washed and the Fbp was purified by CTAB extraction and CM Sepharose chromatography (41). The protein content of each fraction was determined by the A_{280}, and ^{59}Fe was quantitated by liquid scintillation spectrometry. Fractions 4 to 14 were pooled, analyzed by SDS-PAGE and immunoblotting with anti-Fbp sera, and confirmed to contain Fbp (inset within figure).

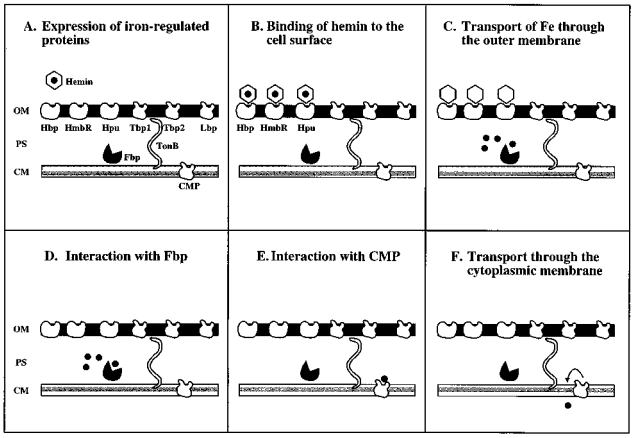

FIG. 4. Model for the acquisition of hemin by *N. gonorrhoeae*. (A) *N. gonorrhoeae* senses conditions of iron and/or hemin limitation and responds by expressing several iron-repressible proteins, including Tbp1, Tbp2, and Lbp (10, 16), and putative hemin-binding proteins (Hbp) (34). Although homologs have not yet been detected in *N. gonorrhoeae*, we have also included the *N. meningitidis* hemoglobin binding receptor (HmbR) (55) and the *N. meningitidis* hemoglobin-haptoglobin-binding protein (Hpu) (39). CMP, cytoplasmic permease. (B) The gonococcal Hbp and/or the putative gonococcal HmbR and Hpu bind hemin through the PPIX ring. (C) Fe is transported across the outer membrane. Our studies indicate that the Fe is removed from the PPIX ring prior to the entry of Fe into the cell. (D) In the periplasm Fe interacts with the gonococcal ferric binding protein, Fbp. (E) Iron is then removed from the Fbp, and it interacts with a CMP. (F) Fe is released from the CMP as it is transported across the cytoplasmic membrane. The precise molecules participating in each step of the transport process have not all been identified or fully characterized, and they are currently under investigation in several laboratories. OM, outer membrane; PS, periplasmic space; CM, cytoplasmic membrane; TonB, TonB protein (51).

single peak of labeled iron (Fig. 3). The presence of Fbp in these fractions was confirmed by Western blot analysis using polyclonal antisera to Fbp (Fig. 3). Carrying out a similar experiment in the presence of KCN reduced the amount of ^{59}Fe bound by Fbp and inhibited the cellular accumulation of iron (data not shown). These results demonstrate that iron bound to hemin associates with Fbp during the course of uptake and that energy is required for the removal of iron bound to hemin. Thus, in addition to its role in the transport of Fe from TF and Fe-citrate (46), Fbp also appears to function in the transport of Fe from hemin.

Model for hemin uptake in *N. gonorrhoeae*. A hypothetical model for the process of high-affinity iron acquisition from hemin by *N. gonorrhoeae* that is consistent with the results of our binding and transport studies as well as those of previous studies with both *N. gonorrhoeae* and *N. meningitidis* (33–37, 39, 55) is shown in Fig. 4. In this model, an iron-responsive regulator, such as Fur, senses conditions of iron and/or hemin limitation (Fig. 4A); this derepresses the expression of the hemin acquisition machinery, which includes hemin-binding outer membrane components. These may include the gonococcal hemin-binding proteins described by Lee (34) and the *N. meningitidis* hemoglobin receptor described by Stojiljkovic et al. (55), as well as the *N. meningitidis* hemoglobin-haptoglobin receptor recently described by Lewis and Dyer (39). TF and LF receptors are also expressed under conditions of iron limitation; however, our data indicate that the TF receptors may not be directly involved in hemin uptake. We propose that hemin first binds to an outer membrane receptor through the PPIX portion of the molecule (Fig. 4B). Our results indicate that hematoporphyrin and hemoglobin can compete with radiolabeled hemin for binding. In addition, by using [^{14}C]hemin, we detected binding of hemin to the gonococcal cell surface. This suggests that the heme moiety and in particular the PPIX ring could be the ligand recognized by the proposed receptor. Our results also indicate that [^{59}Fe]hemin accumulation, but not [^{14}C]hemin accumulation, can be detected in *N. gonorrhoeae*. Thus, following hemin binding to the cell surface, the Fe from the hemin molecule may be transported into the cell (Fig. 4C); however, the mechanisms involved in the removal of the iron are not known. Once within the cell, iron interacts with the periplasmic binding protein Fbp (Fig. 4D) and then is transported across the cytoplasmic membrane by a cytoplasmic permease (Fig. 4E and F); these steps are analogous to the well-described active transport processes in other gram-negative organisms (48, 61). A putative *N. gonorrhoeae* TonB-like protein would function to translocate energy from the cytoplasmic membrane to the outer membrane during the iron transport process. Reports of TonB-dependent OM receptors in *N. gonorrhoeae* and *N. meningitidis* (9, 39, 55) coupled with the results presented here support the existence of a TonB analog in the pathogenic *Neisseria* spp. However, we should stress that with the exception of Fbp (46), the *N. meningitidis* hemoglobin and hemoglobin-haptoglobin receptors (39, 55), and the putative *N. gonorrhoeae* hemin-binding proteins (34), the specific *N. gonorrhoeae* molecules participating in each of the steps in hemin transport have not been identified.

DISCUSSION

The pathogenic *Neisseria* spp. possess several mechanisms for obtaining essential iron from their environment. These mechanisms differ from the typical siderophore-mediated mechanism described for many other pathogens. For example, gonococci express both TF and LF receptors to acquire iron directly from these host binding proteins. The TF receptor appears to consist of a complex of two TF-binding proteins, the relatively conserved Tbp1 and the antigenically and size variable Tbp2. Although Tbp2 is required for TF-mediated iron uptake, its TF binding activity appears to depend on the presence of Tbp1 (2, 15, 16). Lbp1 has been shown to bind LF in vitro; recent studies using an *N. gonorrhoeae* insertional mutant with a mutation in the Lbp1 gene (*lbpA*) have confirmed the role of Lbp1 in LF binding and utilization (10). In addition to the utilization of TF- and LF-bound iron, *N. gonorrhoeae* and *N. meningitidis* can utilize free hemoglobin and hemoglobin bound to haptoglobin and free heme (17). By use of hemin-agarose in batch affinity chromatography, putative hemin-binding proteins of 97 and 50 kDa and 97 and 44 kDa have been isolated from *N. meningitidis* and *N. gonorrhoeae*, respectively (34, 35). Biotinylated human hemoglobin has also been used as an affinity ligand to identify putative hemoglobin-binding proteins from *N. meningitidis* (36). Interestingly, by this approach proteins with molecular weights identical to those of the putative hemin-binding proteins were isolated, suggesting similar mechanisms of hemin and hemoglobin utilization. FrpB, an iron-regulated neisserial protein, has been suggested to play a role in hemin utilization; however, a function for this protein in iron uptake has not been documented (9). A putative haptoglobin-hemoglobin-binding protein (Hpu) in *N. meningitidis* has also been identified and proposed to be involved in hemoglobin utilization (39). The haptoglobin-hemoglobin receptor appears to specifically recognize the heme moiety as a ligand. Stojiljkovic et al. (55) have also recently cloned and characterized an *N. meningitidis* hemoglobin binding receptor, HmbR, which has homology with the TonB-dependent outer membrane receptors of gram-negative bacteria. These authors also found that the mechanism of hemin internalization via the *N.*

meningitidis hemoglobin receptor was TonB dependent in *E. coli.*

In this study, we have characterized the transport of hemin by *N. gonorrhoeae* and defined the specificity of the gonococcal hemin receptor. The ability of hematoporphyrin to inhibit binding and uptake of ^{59}Fe from radiolabeled hemin suggests that the presence of iron within the tetrapyrrole ring is not required for its recognition as a ligand by the putative hemin receptor. The ability of hemoglobin or hematoporphyrin to compete with binding of ^{59}Fe from radiolabeled hemin suggests that a common receptor which recognizes hemin (through the PPIX ring) may be involved in binding of both hemin and hemoglobin. Thus, *N. gonorrhoeae* may utilize at least two receptors for the binding of heme-containing compounds. One is the putative hemoglobin receptor, which allows binding of both hemin and hemoglobin, while the second receptor is specific only for hemin. This system is analogous to the hemin transport system recently proposed for *N. meningitidis* (55). The existence of a second receptor for hemin binding in *N. meningitidis* was inferred from the isolation of cosmid clones in *E. coli* that allowed a *hemA* mutant to utilize hemin but that were distinct from *hmbR*. Analyses of mutants defective in iron transport also support the hypothesis that different receptors for the transport of Fe from hemin and hemoglobin may exist. Stojiljkovic et al. (55) have also shown that an *N. meningitidis hmbR* mutant that was unable to use hemoglobin retained the ability to utilize hemin as a sole source of iron. This mutant may not be capable of release or internalization of hemin through HmbR, and transport of hemin would presumably occur via a second hemin receptor. We have previously reported the isolation and characterization of a mutant of *N. gonorrhoeae* that could grow with hemin, but not hemoglobin, as an iron source (21). We have also previously described a mutant of *P. gingivalis* which is defective in the ability to transport hemin but has retained the ability to utilize hemoglobin (23). Taken together, these studies support the existence of two hemin binding receptors which function in the transport of hemin and hemin bound to hemoglobin. We cannot rule out the possibility that two distinct receptors may be present, i.e., one specific for hemoglobin and one specific for hemin. The interaction between the putative gonococcal hemoglobin receptor and hemoglobin may result in the release of either iron or hemin. In this regard, binding of hemoglobin to the *N. meningitidis* HmbR has been suggested to cause a conformational change in the hemoglobin which results in the release of hemin from the globin (55). The hemin would then be available to bind to a hemin-binding protein, and this would result in the inhibition of hemin binding and uptake as observed in the experiments reported here.

The inhibition of hemin accumulation observed with TF in our studies may have resulted from the release of iron from TF and subsequent transport into the cell. Since our results also indicate that the system for transport of inorganic iron may be more efficient than that for transport of hemin, we would expect inorganic iron to compete with hemin for transport. The observed inhibition of hemin accumulation by TF could also be due to nonspecific binding of TF to a putative gonococcal HmbR, since the *N. meningitidis* HmbR has been reported to exhibit a high degree of homology with the neisserial TF receptors (55). We also found that hemin had a reduced capacity to compete for binding of ^{59}Fe from radiolabeled hemin compared with hemoglobin when at 1:1 and 1:10 molar ratios. These results indicate that hemoglobin may be a preferred heme-iron source compared with hemin. Interestingly, Lee and Hill (36) also found that hemin exhibited a diminished capacity relative to human and bovine hemoglobin and bovine catalase to inhibit biotinylated hemoglobin probe binding competitively to *N. meningitidis.*

Our results also indicate that in *N. gonorrhoeae*, iron is removed from the PPIX ring prior to the entry of Fe into the cytoplasm of the cell. However, we should stress that further studies are required to determine exactly where within the cell and precisely how the iron is removed from the PPIX ring. Previous studies with *N. meningitidis* have shown that iron from Fe-citrate is also removed prior to transport into the meningococcal cell and that citrate is not transported into the cell (52). Interestingly, in our studies we observed that Fe-citrate as a source of inorganic iron did not compete for binding of ^{59}Fe from radiolabeled hemin but did compete for hemin uptake. Our data also suggest that the system for the uptake of Fe from hemin may not be as efficient as the system used for the transport of inorganic iron and that hemin transport in *N. gonorrhoeae* may be regulated by iron and Fur.

The results presented here also indicate that once iron is taken up into the cell, it interacts with Fbp. This is consistent with the role of Fbp as a periplasmic binding protein that functions to shuttle iron from the periplasm through the cytoplasmic membrane. The existence of Fbp analogs in members of the families *Moraxellaceae* and *Enterobacteriaceae* and the genera *Haemophilus* and *Serratia* has been reported (1, 3). Interestingly, two genes downstream of the *N. gonorrhoeae fbp* gene have recently been identified (*nfuB* and *nfuC*), and they appear to compose an iron uptake operon in the gonococcus (19, 44): this operon is homologous to the previously described periplasmic iron uptake operons in *S. marcescens* and *H. influenzae* (1, 3), with *nfuB* coding for a hydrophobic protein (NfuB) and *nfuC* coding for an ATP-binding protein (NfuC). Taken together, these results suggest that the iron transport process mediated by Fbp may represent a common theme in the iron acquisition mechanisms of gram-negative pathogens (61). The promoter region of the *N. gonorrhoeae fbp* gene has two regions that exhibit homology with the *E. coli* consensus Fur-binding sequences (19, 30), and gonococcal and meningococcal *fur* homologs have recently been identified (8, 32, 56). In previous studies using gel retardation assays, purified *E. coli* Fur was shown to bind to at least two sites within the *fbp* promoter sequence (8); however, we do not presently know which of these sites in the *fbp* promoter has the highest affinity or how these sites function in vivo. Putative Fur-iron complex binding domains also precede the putative iron-regulated *N. meningitidis* cytotoxin-encoding genes *frpA* and *frpC*, the gonococcal and the meningococcal *lbpA*, the gonococcal and meningococcal *fur* genes, and the neisserial Tbp2 gene (*tbpB*), which is arranged in tandem in the genome with the Tbp1 gene (*tbpA*) (8, 10, 15, 50, 56–58). Together, these observations have led to the hypothesis that the pathogenic *Neisseria* spp. regulate the expression of iron-regulated proteins through a Fur-like mechanism.

Several pathogenic bacteria are able to use heme and hemoproteins as iron sources by mechanisms involving outer membrane heme-binding proteins and heme transport systems (48). A number of hemin and hemoprotein receptors have been identified by both biochemical and genetic techniques. By ligand chromatography putative hemin receptors have been detected in *H. influenzae* and *P. gingivalis* (20, 33). In addition, hemin and hemoprotein receptors have been identified by cloning of the corresponding genes in *H. influenzae* (*hbpA* and *hxuABC*), *V. cholerae* (*hutA*), *Y. enterocolitica* (*hemR*), *Y. pestis* (*hmsRFH*), and *S. marcescens* (*hasA*) (14, 25–29, 38, 53, 55). Recently, a complete hemin-specific periplasmic-binding-protein-dependent system operon in *Y. enterocolitica* (the *hemSTUV* operon) has been cloned and characterized (53). In this system,

HemT serves as the periplasmic binding protein, HemU is the hydrophobic component of the system, HemV is an ATP binding component, and HemS is probably involved in the degradation of hemin in the cytoplasm. The hemin transport system in *N. gonorrhoeae* may be similar to the *Y. enterocolitica* system, with Fbp serving as the periplasmic binding component, NfuB serving as the hydrophobic component, and NfuC serving as the cytoplasmic ATP binding component (19, 44).

The observed requirement for energy for the accumulation of hemin by *N. gonorrhoeae* suggests that a TonB homolog may function to couple energy across the cytoplasmic membrane. The TonB protein has recently been shown to be required for the transport of hemin in *Y. enterocolitica* and *H. influenzae* (31, 54). Interestingly, the *N. gonorrhoeae* iron-regulated protein FrpB has recently been shown to have homology with several TonB-dependent outer membrane receptors of *E. coli*, as well as with HemR, the heme receptor of *Y. enterocolitica* (9). The gonococcal Tbp1 and meningococcal HmbR also have homology with the TonB outer membrane protein receptors of *E. coli* (15, 55). Preliminary sequence analysis of the 5′ region of the *N. meningitidis* Hpu gene also indicates that Hpu belongs to the TonB-dependent high-affinity receptor family (39). Although these studies indicate that a TonB analog may be present in the pathogenic *Neisseria* spp., no such protein has been identified to date.

As with other pathogens, a requirement for the in vivo growth of *N. gonorrhoeae* is that the organism be capable of obtaining iron from the host. The majority of studies of the mechanism of iron acquisition in the gonococcus have focused on utilization of TF- and LF-bound iron. However, little is known about the mechanisms of hemin transport in this organism. The studies presented here indicate that hemin binds to a gonococcal outer membrane receptor through the PPIX portion of the molecule and that following binding iron is removed and transported into the cell. Our results also indicate that the periplasmic binding protein Fbp is involved in the binding of iron from hemin. Further studies to identify additional proteins involved in gonococcal hemin transport as well as to determine the fate of the PPIX ring are currently in progress.

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