PURIFICATION AND CHARACTERIZATION OF THE MAJOR IRON-REGULATED PROTEIN EXPRESSED BY PATHOGENIC *NEISSERIAE*

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Specific systems responsible for withholding growth-essential nutrients from invading pathogens confer nonspecific protection against bacterial infection. This concept of nutritional immunity was suggested by Kochan (1) in reference to the ability of the human host to sequester free iron. Vertebrates have evolved an elaborate, yet remarkably efficient strategem to withhold growth-essential iron from microbial and neoplastic invaders while retaining their own access to this metal (2). In biological fluids (serum and secretions) free extracellular iron is bound to the host iron-binding proteins transferrin and lactoferrin (3). The amount of free iron that remains in equilibrium with these proteins is on the order of 10^{-18} M, far too low to support bacterial growth (4). During the course of a natural infection, pathogenic bacteria must encounter and overcome this iron-restricted environment. To do so, microorganisms express high-affinity iron-uptake systems that can compete with transferrin and lactoferrin for iron (1–5). These systems are iron-repressible and use proteins as integral components for iron sequestration (5). For example, the pathogens *Escherichia coli* and *Vibrio anguillarum* require that unique proteins be expressed under conditions of iron limitation for their high-affinity iron-uptake systems to function (6). The presence of an efficient iron-uptake system in these organisms is correlated with their ability to cause disease (6). These examples suggest that proteins expressed under conditions of iron limitation (iron-regulated proteins) serve pathogenically relevant roles during the course of infection and warrant consideration when dissecting the pathobiology of disease-producing microbes.

The pathogenic species of the genus *Neisseria, N. gonorrhoeae* and *N. meningitidis*, express several new proteins when grown under iron-limiting conditions. Norqvist et al. (7) originally described the presence of multiple high-molecular-weight gonococcal iron-regulated proteins (70,000–100,000); the number and apparent molecular weights of these proteins varied between isolates examined

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(7–9). Similarly, proteins in the same molecular weight range are expressed by meningococci when grown under iron-limiting conditions (10). The molecular mass of these neisserial proteins are similar to the iron-regulated proteins expressed by other Gram-negative organisms and may serve analogous roles in iron acquisition (7).

A lower molecular weight iron-regulated protein (36,000–37,000) is expressed by all gonococcal isolates (8, 9). This protein appears to be structurally conserved and is a quantitatively major component of the total protein repertoire when gonococci are grown under iron-limiting conditions (9). An antigenically cross-reactive iron-regulated protein is similarly expressed by *N. meningitidis*, as well as by two nonpathogenic *Neisseria* species (*N. lactamica* and *N. cinerea*) (11). Thus, this protein is referred to as the major iron-regulated protein (MIRP)[1] of the neisserial strains expressing it. The highly conserved nature of this protein implies that some essential function is being served by its presence. It is tempting to speculate that this function is a component of a high-affinity iron-uptake system. However, to date neither this nor any other function has been ascribed to the MIRP.

To facilitate studies on the functional and pathogenic roles of the MIRP, a procedure to purify this protein in quantities sufficient to permit biological and chemical analysis was developed. This procedure and several of the biochemical characteristics of the MIRP are described in this report.

Materials and Methods

Media and Reagents. Gonococcal (GC) agar medium (Difco Laboratories Inc., Detroit, MI) supplemented with 1% (vol/vol) growth factor (12) and 0.5% (wt/vol) glucose was used for the routine maintenance of all organisms. Cultures of *N. gonorrhoeae* and *N. meningitidis* were grown at 37°C in a humidified atmosphere containing 4% CO_2. For propagation of organisms under conditions of iron limitation, we used the iron chelator Desferal mesylate (Ciba-Geigy Corp., Summit, NJ) at a final concentration of 25 μM to supplement the GC agar medium described above. *N. gonorrhoeae* and *N. meningitidis* are unable to remove iron bound to Desferal (13), and this medium (referred to as low-iron medium) has been used previously (11) to induce expression of the gonococcal and meningococcal MIRP.

SDS, acrylamide, 2-ME, urea, molecular weight standards for SDS-polyacrylamide gels, and bromphenol blue were obtained from Bio-Rad Laboratories, Richmond, CA. Tris (hydroxymethyl)-aminomethane base (Tris), hexadecyltrimethylammonium bromide (CTB), Triton X-100, PMSF, transferrin, and standard iron stock solution were obtained from Sigma Chemical Co., St. Louis, MO. Hepes was purchased from Research Organics Inc., Cleveland, OH; Coomassie Brilliant Blue (No. G250) (Coomassie Blue) was from Bethesda Research Laboratories, Bethesda, MD; and Zwittergent 3-14 was from Calbiochem-Behring Corp., La Jolla, CA. Carboxymethyl(CM)–Sepharose CL-6B, Sephacryl S-300, gel filtration molecular weight standards, and IEF standards were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. All other chemicals were of reagent grade.

Bacteria and Growth Conditions. *N. gonorrhoeae* strain F62 was provided by R. P. Williams, Baylor College of Medicine, Houston, TX. Transparent, nonpilate morphotypes of this strain were selected for subsequent propagation. Initial conditions for the purification of the MIRP were established using this strain as the source of protein. This purification scheme was subsequently applied to *N. meningitidis* strain 80084313 (serogroup A) obtained from R. J. Arko, Sexually Transmitted Diseases Laboratory Program,

[1] *Abbreviations used in this paper:* CM, carboxymethyl; CTB, hexadecyltrimethylammonium bromide; GC, gonococca MIRP, major iron-regulated protein; Pth, phenylthiohydantoin.

Centers for Disease Control, Atlanta, GA. Inocula of either strain were prepared after growth on GC agar medium for 20 h. Cells were removed with a dacron swab and streaked heavily onto large petri plates (150 mm × 15 mm) containing 25 ml of low-iron GC agar medium. The inoculated plates were incubated for 16–20 h, at which time cells were harvested by suspending the growth from the agar plates in Davis A minimal medium (Difco Laboratories, Inc.) to remove soluble contaminants contributed by the media from the organisms.

Cell Fractionation. Bacteria harvested from 2 liters of low-iron agar medium (80 plates) were suspended in 100 ml of 10 mM Hepes buffer, pH 7.4, containing 0.1% (vol/vol) protease inhibitor (10 mM PMSF in isopropanol). Aliquots of 5 ml were disrupted by sonication at 75 W for a total of 1 min (4°C) using a high intensity sonifier equipped with a microtip (Branson Instruments Inc., Stamford, CT). This suspension was centrifuged at 48,000 *g* for 60 min. The pellet was enriched for membranes (referred to as the particulate fraction), while the nonsedimentable supernatant (referred to as the soluble fraction) contained primarily soluble proteins.

Selective Solubilization of the MIRP from the Particulate Fraction. The MIRP associated with the particulate fraction was selectively solubilized using the detergent CTB. The optimum detergent/protein ratio was determined for the particulate fraction isolated from *N. gonorrhoeae* as follows. The particulate fraction was washed once in 10 mM Hepes buffer, pH 7.4, containing protease inhibitor, subjected to centrifugation (48,000 *g* for 60 min at 4°C), and the pellet was then suspended in 10 mM Tris buffer, pH 8.0, to a final protein concentration of 1 mg/ml. Increasing amounts of CTB, to a final concentration of between 0.0 and 0.8% (wt/vol), were added to 1-ml aliquots of this suspension, and the mixtures were incubated at room temperature for 20 min. The insoluble material was removed by centrifugation (48,000 *g* for 60 min at room temperature). Equal volumes of the supernatants were analyzed by SDS-PAGE for the relative proportion of the MIRP released by exposure to the respective concentration of CTB. The optimal detergent/protein ratio was defined as the lowest concentration of CTB that solubilized the maximal amount of the MIRP associated with the particulate fraction. This value was used for selective solubilization of both the meningococcal and gonococcal MIRPs.

Preparation of Crude MIRP Extracts. The pellet from sonicated whole organisms was diluted to a final protein concentration of 1 mg/ml and CTB was added to the optimal detergent/protein ratio. After incubation for 20 min at room temperature, insoluble material was removed by centrifugation (48,000 *g* for 60 min at room temperature). The supernatant was enriched for the MIRP and was referred to as the crude detergent extract of the membrane-associated MIRP.

A similar method was used to obtain a preparation enriched for the MIRP from the soluble fraction of sonicated whole cells. This fraction was diluted to a final protein concentration of 1 mg/ml in 10 mM Tris buffer, pH 8.0. The addition of CTB (0.05%, wt/vol, final concentration) resulted in the formation of a white precipitate. This mixture was stirred at room temperature for 20 min and the precipitate was removed by centrifugation (48,000 *g* for 60 min at room temperature). The resulting supernatant was enriched for the MIRP and is referred to as the crude detergent extract of the soluble MIRP.

Ion-exchange Chromatography. Proteins contained in the crude detergent extracts were separated by ion-exchange chromatography with the cation-exchange gel matrix CM–Sepharose 6B-CL. 35 ml of matrix slurry were poured into a 250 × 20 mm column and washed sequentially with two bed-volumes of 0.1 N NaOH and two bed-volumes of 10 mM Tris buffer (pH 8.0) containing 1 M NaCl and 0.05% (wt/vol) CTB. The column was then equilibrated with one bed-volume of 10 mM Tris buffer (pH 8.0) containing 0.05% (wt/vol) CTB (Tris/CTB buffer). The crude detergent extracts of the MIRP were applied to the column in Tris/CTB buffer and the matrix was washed with the same buffer until all material not interacting with the column had eluted. A 0–1 M NaCl gradient of Tris/CTB buffer in a total volume of 500 ml was used to elute proteins from the column. The column flow rate was maintained at ~25 ml/h and protein elution was monitored by absorbance at 280 nm with an in-line UV monitor. Fractions of 4 ml were

collected and the appropriate fractions were pooled. Fresh column packing was used for each purification.

Molecular Sieve Chromatography. MIRP-containing fractions from the ion-exchange column were further purified based on molecular mass using a 850 × 20 mm Sephacryl S-300 column equilibrated with Tris/CTB buffer containing 0.3 M NaCl. These fractions were applied in a total volume of 2 ml and chromatographed at a flow rate of 30 ml/h. Protein elution was monitored in-line by absorbance at 280 nm. The molecular mass of the MIRP was deduced by comparison to the elution characteristics of the following set of molecular weight standards: ferritin, 440,000; catalase, 232,000; BSA, 67,000; OVA, 43,000; chymotrypsinogen A, 25,000; and ribonuclease A 13,600.

SDS-PAGE. SDS-PAGE was performed using the gel and buffer formulations described by Laemmli (14). The resolving gel consisted of 10% acrylamide (wt/vol) and contained 70 mM NaCl as described previously (8). All protein determinations were performed using the method of Markwell et al. (15). Before electrophoresis, protein concentrations were adjusted to 1 mg/ml with a final sample buffer consisting of 6.25 mM Tris buffer, pH 6.8, 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.001% (wt/vol) bromphenol blue, and 5% (vol/vol) 2-ME. Samples were heated to 100°C and held for 5 min. Electrophoresis was carried out using slab gels that were 140 mm long and 1 mm thick. A constant current was applied and electrophoresis was terminated when the dye front reached the bottom of the gel. The gels were stained for 2–12 h in a solution of 0.1% (wt/vol) Coomassie Blue in water/methanol/acetic acid (5:5:2). A 10% (vol/vol) acetic acid solution was used to destain the gels. Alternatively, gels were stained with silver by the method of Tsai and Frasch (16) modified by the omission of the periodate oxidation step.

Isoelectric Focusing. Isoelectric focusing was performed using previously described methods (17). Protein samples were dialyzed against 10 mM Tris buffer (pH 7.2) containing 6 M urea and 0.01% Triton X-100. Prepoured native polyacrylamide gels containing an ampholine mixture of 5.5–9.5 pI range (LKB Produkter, Bromma, Sweden) were used in this analysis. Isoelectric points were extrapolated from a mixture of standard proteins with known pI values.

Amino Acid Composition and H_2-Terminal Sequence Analysis. Purified MIRP fractions were prepared for this analysis by dialysis for 48 h at 4°C against five changes of distilled water. This manipulation resulted in the formation of a precipitate, which was removed by centrifugation. After dialysis and centrifugation, ~60% of the predialysis protein was recovered. The amino acid composition was determined after hydrolysis in 6 N HCl (18) in evacuated sealed tubes at 115°C for 22, 48, 72, and 96 h. The values for serine and threonine were corrected for destruction during hydrolysis by extrapolation to time 0. The values for leucine, isoleucine, and valine were corrected for slow hydrolysis of the peptide bond by extrapolation to infinite time. Half-cystine and methionine were determined as cysteic acid and methionine sulfone, respectively, after performic acid oxidation.

Automated Edman degradation was performed with a sequenator (No. 890C; Beckman Instruments, Inc., Palo Alto, CA), using a modified Quadrol program (No. 001576; Beckman Instruments, Inc.) in combination with polybrene. Thiazolinone derivatives of amino acids were converted to phenylthiohydantoin (Pth)-derivatives with aqueous 1.0 N HCl at 80°C for 10 min. Pth–amino acids were identified by HPLC and confirmed by gas chromatography and/or TLC.

Iron Determination. Purified protein preparations including the gonococcal and meningococcal MIRP, the gonococcal protein I (purified by the method of Blake and Gotschlich [19]), and transferrin were analyzed for the presence of iron using the chromogenic method described by Campbell and Zerner (20). Iron concentrations were quantitated by comparison to a stock iron standard (8.95 mM in 0.8 N nitric acid) obtained from Sigma Chemical Company. The net amount of iron associated with each preparation was determined after subtracting the iron concentration contributed by the buffer in which the purified protein preparations were suspended. Protein concentrations for this analysis were determined by the method of Markwell et al. (15). Fractions collected from ion-exchange chromatography of the meningococcal MIRP were assessed for iron content

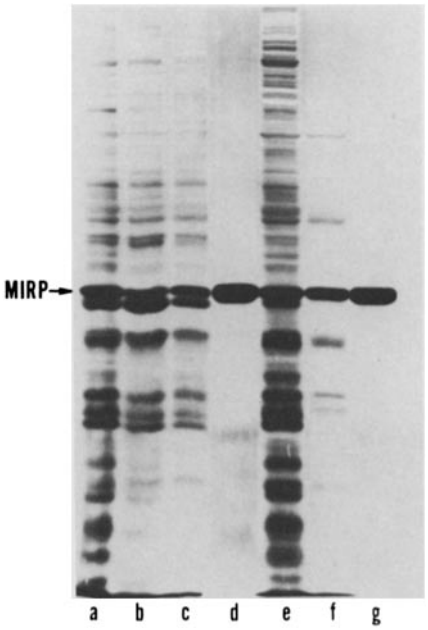

FIGURE 1. Silver-stained, SDS-PAGE analysis of fractions obtained during the purification of the gonococcal MIRP. Whole iron-starved gonococci (*a*) were sonicated and the particulate (*b*) and soluble (*e*) fractions were separated by centrifugation. The particulate fraction was suspended in a volume identical to that of the soluble fraction before SDS-PAGE analysis and 30 μl were electrophoresed per lane. The soluble and particulate fractions were diluted to a protein concentration of 1 mg per ml in 0.01 M Tris buffer, pH 8.0, containing 0.05% CTB (wt/vol), they were incubated at room temperature, and the insoluble material was removed by centrifugation. (*c* and *f*) 30 μl of CTB extracts from the particulate and the soluble fractions of whole cell sonicates, respectively. (*d* and *g*) MIRP-containing fractions (10 μg of protein) isolated by ion-exchange chromatography from the particulate and soluble fractions of whole cell sonicates, respectively.

by analyzing 100 μl of each fraction. The elution of protein during ion-exchange chromatography was monitored by absorbance at 280 nm. All glassware used in this procedure was acid washed before use.

Results

Purification

Cellular Disruption and Fractionation. Iron-starved gonococci were harvested, washed, subjected to sonication, and the soluble and particulate fractions were separated by centrifugation as described. Analysis by SDS-PAGE indicated that the MIRP was approximately equally distributed in each fraction (Fig. 1, *b* and *e*). The MIRP associated with the soluble fraction could not be removed by centrifugation at 60,000 *g* for 90 min (data not shown), suggesting that it was not membrane bound. Conversely, the MIRP from the particulate fraction appeared to be tightly membrane bound or stably aggregated as subsequent washings of the pellet did not remove detectable amounts of this protein (data not shown).

Extraction of the Particulate and Supernatant Fractions with CTB. Initial observations indicated that the MIRP was completely solubilized from the particulate fraction in the presence of 2% (wt/vol) CTB. By contrast, other proteins in the crude membrane fraction were only partially solubilized. Therefore, a series of lower CTB concentrations were analyzed for their potential to selectively solubilize the MIRP from the particulate fraction. The experiment depicted in Fig. 2 demonstrates the effect of CTB concentration (0.0–0.8%, wt/vol) on the release of the MIRP from the particulate fraction. The lowest concentration of CTB that released the maximal amount of the MIRP was 0.05% (wt/vol). This value is slightly larger than the critical micell concentration of CTB at low ionic

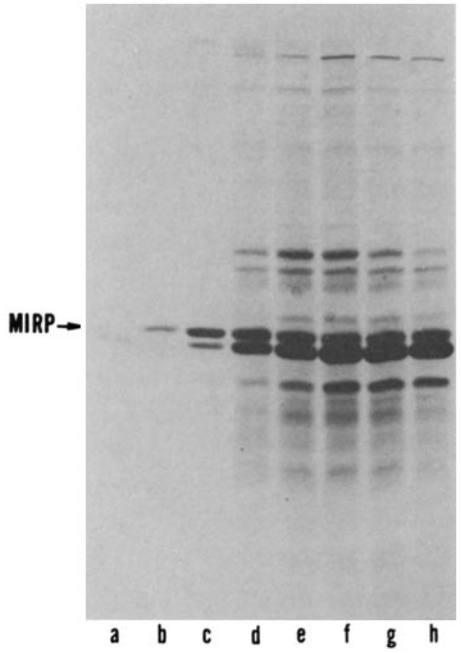

FIGURE 2. Selective solubilization of the gonococcal MIRP from the particulate fraction of whole cell sonicates in the presence of increasing concentrations of CTB. Aliquots of membrane-containing fractions (1 mg/ml) were incubated with CTB at detergent/protein ratios (wt/wt) of 0 (*a*), 0.125 (*b*), 0.25 (*c*), 0.5 (*d*), 1.0 (*e*), 2.0 (*f*), 4.0 (*g*), and 8.0 (*h*). After a 20 min incubation at room temperature, the insoluble material was removed by centrifugation. Equal volumes (40 μl) of the solubilized material were electrophoresed, stained with Coomassie blue, and assessed for the relative quantities of MIRP released from the particulate fraction. The MIRP was preferentially solubilized at a detergent/protein ratio of 0.125 and 0.25. Maximal amounts of the MIRP were solubilized from the particulate fraction at a ratio of 0.5.

strength (21), and represents a detergent/protein ratio of 0.5 (wt/wt). These conditions were subsequently used to extract the MIRP from the particulate fraction (Fig. 1 *c*).

The same detergent/protein ratio was added to the supernatant fraction from sonicated whole cells. Upon addition of the CTB, a white precipitate formed which could be removed by centrifugation. The resulting supernatant was enriched for the MIRP (Fig. 1 *f*).

Purification of the MIRP from CTB Extracts. The MIRP was further purified by ion-exchange chromatography using a cation-exchange matrix (CM-Sepharose 6B-CL). The CTB-extracts were applied to the ion-exchange column in Tris/CTB buffer. The majority of proteins did not bind to the column matrix and were removed by washing with Tris/CTB buffer. The first major peak that resolved when a linear (0–1.0 M) NaCl gradient was applied to the column (Fig. 3) eluted with a characteristic pink color. Silver-stained SDS-polyacrylamide gel analysis revealed that fractions corresponding to this peak contained the MIRP as the predominant protein (Fig. 1, *d* and *g*). No differences in the elution profiles were seen between the MIRP isolated from the supernatant or particulate fractions of the whole cell sonicates. Therefore, in subsequent purifications the CTB extracts from both fractions of the whole cell sonicates were pooled before ion-exchange chromatography. A diffuse peak associated with a component of the CTB eluted at a much higher NaCl concentration; consequently, fractions were collected only through the first half of the NaCl gradient. Residual proteins contaminating the MIRP-containing ion-exchange fractions were removed by gel filtration chromatography using Sephacryl S-300 (Fig. 4). After this step, the MIRP-containing fractions appeared to be pure by the criterion of a single band by SDS-PAGE visualized with a sensitive silver stain (Fig. 5, *a* and *b*). Initial

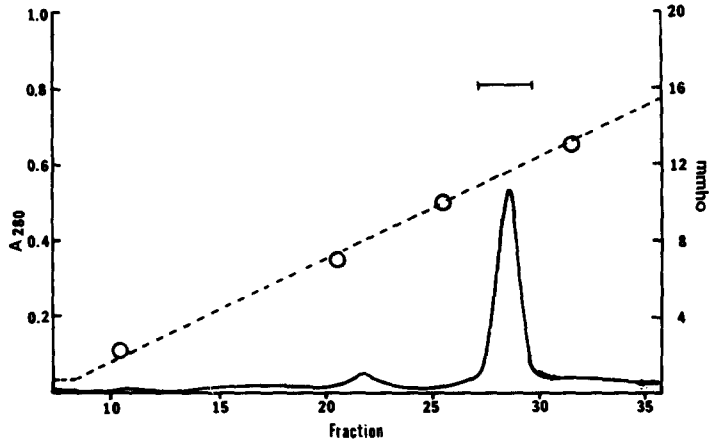

FIGURE 3. Cation-exchange chromatography of pooled CTB extracts from the particulate and soluble fractions of whole cell sonicates from gonococci grown in low-iron medium (see Fig. 1, *c* and *e*). The sample was applied to a CM-Sepharose CL-6B column and the bound proteins were eluted using an ascending, linear 0–1.0 M gradient of NaCl (– –○– –). Fractions were monitored for absorbance at 280 nm (——). The bar indicates the fractions that were pooled. The MIRP-containing fractions eluted as a single peak at a conductivity of 11 mmho, which corresponds to a NaCl concentration of 150 mM.

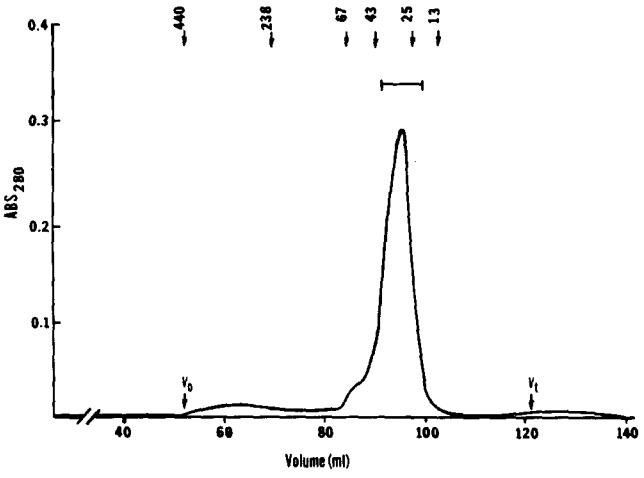

FIGURE 4. Molecular sieve purification of the MIRP-containing fractions obtained from ion-exchange chromatography (Fig. 3). Fractions containing the MIRP were concentrated by lyophilization, and separated from contaminating proteins using the gel permeation matrix Sephacryl S-300. The MIRP eluted with a K_{av} of 0.6, which corresponds to an estimated molecular mass between 26,000 and 32,000. The MIRP-containing fractions, indicated by the bar were pooled and analyzed for purity by SDS-PAGE (Fig. 5). Mol wt $\times 10^{-3}$ are shown.

purification steps (described above) were established using *N. gonorrhoeae* as the source of the MIRP. Subsequently, conditions used for purification of the gonococcal MIRP were applied without modification to the isolation of the meningococcal MIRP (Fig. 5, *c* and *d*).

Yield. Typically, 10–20 mg of the purified MIRP could be isolated from organisms grown on 2 liters (80 plates) of low-iron GC agar medium. The yield was positively correlated with the degree of iron deprivation. When maximally expressed, the purified MIRP composed between 1–3% of the total protein content of gonococci or meningococci.

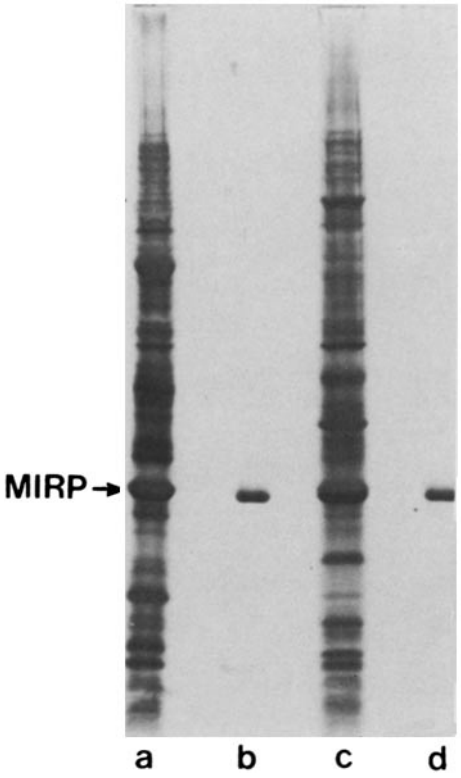

FIGURE 5. Silver-stained, SDS-PAGE analysis of whole cell sonicates and the purified MIRP after extraction with CTB, cation-exchange chromatography, and gel filtration chromatography. (*a* and *c*) Total whole cell sonicates of *N. gonorrhoeae* and *N. meningitidis* grown under conditions of iron limitation, respectively. (*b* and *d*) 10 μg of the purified MIRP from *N. gonorrhoeae* and *N. meningitidis*, respectively.

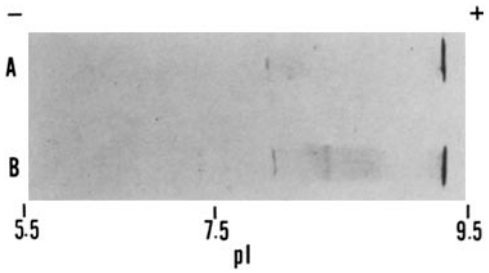

FIGURE 6. Isoelectric focusing of the purified MIRP (20 μg) from the soluble (*A*) and the particulate (*B*) fractions of sonicated whole cells. Both proteins migrate to identical positions near the cathode. Under the conditions used, an isoelectric point of slightly greater than that for the protein standard trypsinogen (pI 9.35) was observed.

Biochemical Characterizations

Molecular Mass. Estimates of molecular mass were deduced from gel filtration chromatography based on the elution of the MIRP relative to protein standards with known molecular weights. The MIRP purified from either gonococci or meningococci eluted with a Kav of 0.6 in Tris/CTB buffer containing 300 mM NaCl (Fig. 4), corresponding to a molecular mass of between 26 and 32 kD.

Isoelectric Point. The purified MIRP isolated from *N. gonorrhoeae* was analyzed by isoelectric focusing using an ampholyte gradient of 5.5–9.5 (Fig. 6). Preparations purified from either the soluble or particulate fractions of whole cell sonicates migrated with identical isoelectric points corresponding to a value slightly greater than the most basic standard, trypsinogen (pI 9.35). The potential contribution of the cationic detergent CTB to the basic isoelectric point of the

TABLE I

Integral Amino Acid Composition of the Gonococcal and Meningococcal MIRPs

Amino acid	Gonococcal MIRP*	Meningococcal MIRP*	Gonococcal protein I‡	Gonococcal protein II‡
Neutral	216 (0.64)§	217 (0.63)	206 (0.67)	163 (0.62)
Aliphatic	176 (0.52)	181 (0.52)	162 (0.53)	147 (0.56)
Gly	27	24	42	32
Ala	54	54	27	26
Val	34	33	27	29
Leu	34	33	18	15
Ile	14	12	6	11
Ser	4	10	27	21
Thr	9	14	15	13
Aromatic	19 (0.06)	17 (0.005)	27 (0.09)	10 (0.04)
Phe	10	10	12	5
Tyr	9	7	15	5
Sulfur-containing	4 (0.01)	3 (0.01)	5 (0.02)	5 (0.02)
1/2 Cys	1	—	1	—
Met	3	3	4	5
Imino acids	17 (0.05)	16 (0.05)	12 (0.02)	1 (0.001)
Pro	17	16	12	1
Charged	124 (0.36)	130 (0.37)	99 (0.32)	99 (0.38)
Dicarboxylic acids	76 (0.22)	74 (0.21)	69 (0.23)	58 (0.22)
Asp/Asn	33	32	33	42
Glu/Gln	43	42	36	16
Basic	48 (0.14)	56 (0.16)	30 (0.10)	41 (0.16)
His	6	7	6	4
Arg	15	15	6	22
Lys	27	34	18	15

* Predictions based upon a molecular mass for the MIRPs of 36 kD as determined by SDS-PAGE.
‡ Data obtained from Blake and Gotschlich (19, 22).
§ Values in parentheses represent the proportion of residues belonging to the chemical category listed in the left-hand margin.

MIRP was considered. This possibility was discounted by the finding that whole gonococci propagated in low-iron medium and solubilized with the nonionic detergent Triton X-100 contained a protein that comigrated with the purified MIRP (data not shown), suggesting that the basic isoelectric point is not likely due to the bound CTB.

Amino Acid Composition. The amino acid composition of the gonococcal and meningococcal MIRPs is shown in Table I. Also included in this Table for comparison are the previously published amino acid compositions of the gonococcal outer membrane proteins, protein I (19) and protein II (22). The amino acid compositions of the meningococcal and gonococcal MIRP molecules were nearly identical and were similar in character to those of protein I and protein II (i.e., the proportion of aliphatic, aromatic, sulfur-containing, and dicarboxylic acid residues being similar). However, the distribution of individual amino acids was unique. Notably, the MIRP contained fewer aromatic amino acids and more proline residues relative to proteins I and II. Furthermore, a higher proportion of basic residues was found for the MIRP in comparison to protein I (a protein

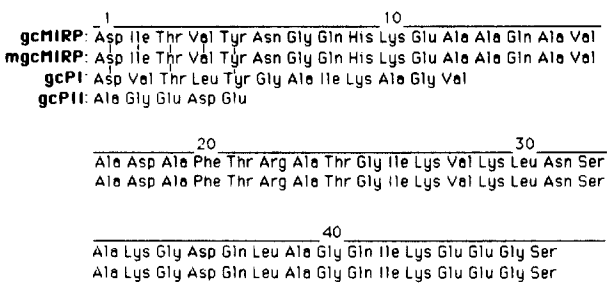

FIGURE 7. NH_2-terminal amino acid sequence through residue 48 of both the gonococcal (*gc*) and meningococcal (*mgc*) MIRP preparations. The published NH_2-terminal amino acid sequences of protein I (*PI*) from the gonococcal strain R10 (19) and protein II (*PII*) from the same strain (22) are shown for comparison. After alignment of NH_2-terminal sequences, residues from the MIRP that matched identically with protein I are indicated with a solid line.

TABLE II
Iron Content of Purified MIRP Preparations

Sample	Protein*	Fe	Fe/Protein
	nM	*nM*	*M/M*
MIRP (gonococcal)	4.1	1.8	0.44
MIRP (meningococcal)	3.9	2.5	0.64
Protein I	3.3	0.0	0.00
Transferrin (iron-saturated)	2.6	3.2	1.23

* Protein concentrations were deduced by the method of Markwell et al. (15) and the molar amounts were determined based upon an estimated molecular mass of 36 kD for the MIRP; 34 kD for protein I; and 74 kD for human transferrin.

exhibiting a relatively acidic isoelectric point [23]). Similarly, protein II also contained a higher proportion of basic amino acids than protein I (22). This property is not surprising since both MIRP and protein II have highly basic isoelectric points and can be purified by cation-exchange chromatography.

NH_2-Terminal Amino Acid Sequence. The NH_2-terminal amino acid sequence of the gonococcal and meningococcal MIRPs are shown in Fig. 7. Both sequences are identical through the first 48 residues. A comparison of these sequences with the published NH_2-terminal amino acid sequences of gonococcal proteins I and II revealed homology with the first five residues of protein I, three being identical and the remaining two representing conservative amino acid substitutions. No other apparent homology between the MIRP sequences and the sequences of protein I or protein II was observed.

Iron Content. The observation that purified MIRP preparations are pink suggested that a metal chromophore might be associated with the protein. This is the case for the basic iron-binding protein lactoferrin which is pink in its ferrated state (24). Given that the MIRP is regulated by the amount of free iron in the medium and that it may play a central role in iron acquisition (8, 9), the iron content of purified MIRP preparations was determined. A molar ratio of iron to protein of ~1:2 was detected for both the meningococcal and gonococcal MIRPs (Table II). For comparison, the iron content of protein I, purified by the method of Blake and Gotschlich (19), was determined. No iron concentrations above the buffer blank were detected in this purified preparation. By contrast,

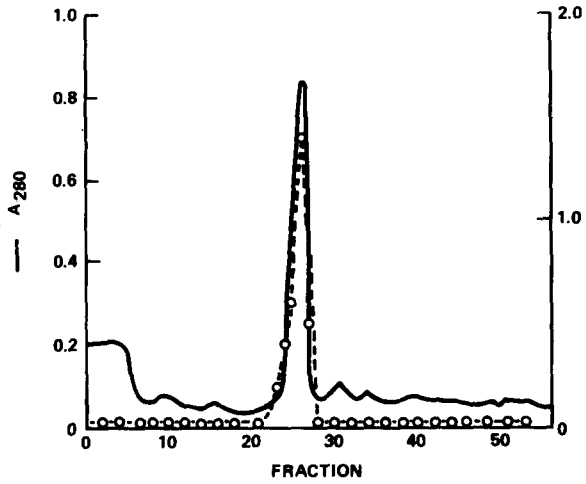

FIGURE 8. Coelution of the meningococcal MIRP and iron from cation-exchange chromatography. CTB extracted MIRP from the particulate and soluble fractions of whole cell sonicates were pooled and subjected to ion-exchange chromatography as described in the text. A linear gradient of 0–1 M NaCl was applied to the column to elute the MIRP. Protein concentrations were monitored by absorbance at 280 nm (——) and the concentration of iron determined for every other fraction (– – –).

ferrated transferrin yielded a molar ratio of iron to protein of 1:1.2. When the iron content of fractions from the cation-exchange chromatographic purification of the meningococcal MIRP was monitored, a single major iron-containing peak eluted at the same position as the MIRP (Fig. 8), providing further evidence that iron is associated with this protein and is not an artifact of the purification procedure.

Discussion

A direct relationship between the ability to obtain iron from the host environment and microbial survival and virulence has been established for certain infectious diseases (1–6). This relationship is suspected to hold for nearly all pathogenic microorganisms (2–4). For pathogenic *Neisseriae*, the critical link between iron acquisition and virulence has been studied to a limited extent in experimental animal models. Payne and Finkelstein (25) reported that virulence of nonpiliated gonococci for chick embryos was enhanced when iron was included in the inoculum, while the virulence of piliated gonococci was not enhanced by iron. Using a mouse model, Holbein et al. (26) found that LD_{50} values for some strains of *N. meningitidis* were decreased as much as 10^9-fold by the addition of iron-dextran to the inoculum. However, the virulence of other meningococcal strains was not affected by simultaneous inoculation with iron. Thus to date, iron-acquisition and virulence remains an attractive but unproven relationship for the pathogenic *Neisseriae*. This is largely a consequence of (*a*) unsuitable animal models for evaluating differences in virulence; (*b*) the unavailability of well-characterized mutants deficient in their ability to obtain iron from the environment; and (*c*) lack of information regarding the mechanism(s) by which gonococci and meningococci obtain iron from the host environment.

The high affinity iron-uptake system of pathogenic *Neisseriae* does not appear to be similar to those that have been characterized for other Gram-negative pathogens (27). The latter use soluble, low-molecular-weight, nonproteinacious siderophores that are secreted into the environment and effectively compete with host iron-binding proteins for iron. Ferrated-siderophore complexes are

subsequently recognized by specific microbial surface receptors and the assimilation of this siderophore-bound iron follows. The presence of an analogous siderophore-mediated, iron-uptake system has not been conclusively demonstrated for the pathogenic *Neisseriae* (9, 28, 29). Moreover, meningococci require direct contact with host transferrin to obtain iron from this molecule (30). A similar observation has been made for the acquisition of lactoferrin-bound iron by *N. gonorrhoeae* (31). These results argue for a surface receptor that allows the organism to obtain iron directly from specific host iron-binding proteins. This is a departure from the soluble siderophore-mediated mechanism of iron acquisition described for other pathogens and may represent a fundamentally different and highly specialized strategy that has evolved in order for obligate mucosal pathogens (such as gonococci and meningococci) to survive within vertebrate hosts. In at least one respect the biology of iron acquisition by pathogenic *Neisseriae* and other Gram-negative bacterial species is similar: Both synthesize novel proteins when iron deprived. Since some iron-regulated proteins of nonneisserial species directly participate in iron acquisition (5), it seems likely that certain iron-regulated proteins expressed by pathogenic *Neisseriae* (in particular the MIRP) may be involved in analogous capacities (8, 9).

To more closely study the functional and pathogenic roles of the MIRP, we have devised a method to efficiently purify this protein in sufficient quantities for chemical and biological analysis. This method involves (*a*) cellular disruption and fractionation by centrifugation; (*b*) enrichment for the MIRP by selective solubilization with the cationic detergent CTB; (*c*) cation-exchange chromatography in the presence of this detergent; and (*d*) gel filtration chromatography. Previous studies have reported the MIRP to be associated with Sarkosyl-insoluble membrane complexes (8), outer membrane blebs (32), and crude membranes (9). Particularly interesting was the finding that upon sonication of cells grown in low-iron medium, the MIRP could be detected in substantial quantities in both the particulate and supernatant fractions (Fig. 1, *b* and *e*). In detergent-free aqueous buffer, the MIRP from the soluble fraction of whole cell sonicates eluted from a gel filtration column with a K_{av} of 0.6 (data not shown), a value identical to the K_{av} of the purified MIRP chromatographed in detergent-containing buffers. This suggests that in aqueous buffers, the MIRP behaves as a monomer and not as a macromolecular aggregate. The nature of the interaction between the MIRP and membranes of the cell envelope as well as the functional significance of this differential partitioning has not been determined.

Blake and Gotschlich (19) used the cationic detergent CTB at high concentrations (2%, wt/vol) and low-ionic strength to precipitate a fraction enriched for protein I from whole gonococci. We determined that all the detectable MIRP is solubilized by these conditions, leading us to analyze lower concentrations of CTB for their potential to selectively solubilize MIRP from gonococcal membranes. Increasing concentrations of CTB (0.0125–0.05%, wt/vol) released the MIRP in a concentration-dependent fashion (Fig. 2). Maximal release of the MIRP was achieved at a concentration of 0.05% (wt/vol), a value that exceeds the reported critical micellar concentration of CTB (21). Given the cationic character of CTB and the basic isoelectric point of the MIRP, it is curious that such a relationship exists, since the two molecules should be subject to ionic

repulsion. Alternatively, evidence from this laboratory indicates that CTB efficiently solubilizes negatively charged membrane phospholipids (Chen, C. Y., personal communication). This effect could indirectly result in the release of the MIRP from the cell envelope.

Addition of CTB to the soluble fraction of whole cell sonicates at a final detergent to protein ratio of 0.5 (wt/wt) resulted in the formation of a white precipitate. The predominant protein species remaining in the cleared supernatants was the MIRP. CTB precipitates acidic polysaccharides under conditions of low-ionic strength (33). Presumably, it is this property that precipitates the nucleic acids, carbohydrates, and associated proteins that are found in the soluble fraction of sonicated whole cells. After centrifugation, the MIRP was retained as the principle protein in the supernatant.

The CTB-solubilized MIRP bound to the ion-exchange matrix CM-Sepharose. The use of cation-exchange chromatography in the presence of cationic detergents is an unusual aspect of this purification scheme. However, the binding of the MIRP was not antagonized by the presence of a positively charged detergent in the buffer and this protein could be eluted as a sharp peak from this column matrix using a linear gradient of NaCl. Analysis of the MIRP-containing fractions by SDS-PAGE revealed contaminating species with apparent molecular masses between 24 and 32 kD. The presence and quantities of these contaminants were variable from preparation to preparation. Blake et al. (22) isolated protein II from gonococci using cation-exchange chromatography. Protein(s) II have molecular masses between 24 and 32 kD (34). Therefore, contaminating protein(s) II may pose a problem when this purification scheme is applied to purification of the gonococcal MIRP. To limit potential copurification of the MIRP and protein II molecules, transparent gonococcal morphotypes were used in the initial inoculum. Meningogoccal MIRP preparations also copurified with minor non-MIRP components, the presence of which varied from preparation to preparation. Contaminating components of both the gonococcal and meningococcal MIRP fractions from ion-exchange chromatography were mitigated by subsequent purification using gel filtration chromatography. Molecular mass estimates obtained by gel filtration indicated that the MIRP eluted as a monomeric species with a molecular mass between 26 and 32 kD. In both aqueous- and detergent-containing buffers, molecular mass estimates were slightly lower but in similar agreement to those obtained by SDS-PAGE analysis (36–37 kD) (8, 9). The micellar molecular mass of CTB has been reported to be 62 kD (21). Since molecular mass estimates by gel filtration chromatography in the presence of this detergent (at concentrations in excess of the critical micelle concentrations) are identical to those obtained in the absence of CTB and in agreement with the subunit molecular mass deduced by SDS-PAGE, it is unlikely that this protein associates with CTB micelles. This is in contrast to the gonococcal protein I and protein II molecules known to interact with Zwittergent 3-14 micelles (19, 22).

The purified meningococcal and gonococcal MIRPs were characterized biochemically with regard to their respective amino acid compositions, NH_2-terminal amino acid sequence, and the amount of iron associated with purified preparations of each. In addition, the highly cationic nature of the gonococcal MIRP was revealed by isoelectric focusing. The difficulties in determining the isoelectric

points for basic proteins are well documented (35, 36). By the methods used in the present study, the gonococcal MIRP purified from both the particulate and soluble fractions of whole cell sonicates appeared to have identical isoelectric points corresponding to a value >9.35, suggesting that the distribution of MIRP as membrane-bound and free is not a function of charge. While the isoelectric point of the meningococcal MIRP was not determined, the pI of this protein is suspected to be similar to the gonococcal MIRP based upon identical purification properties (Fig. 5), amino acid composition (Table I), and NH_2-terminal sequence analysis (Fig. 7). Moreover, given the highly cationic nature of the MIRP, it can be inferred that a substantial number of the aspartate and glutamate residues (Table I) must exist as their respective neutral amides, asparagine and glutamine.

The NH_2-terminal sequence of the gonococcal and meningococcal MIRPs were identical through position 48 (Fig. 7). Thus, the MIRPs of both species appear to be remarkably similar according to molecular mass, charge, amino acid composition, and NH_2-terminal sequence. As reported elsewhere (1), they also exhibit common antigenic determinants. This homology indicates that the MIRP is among the most conserved neisserial molecules of pathogenic significance. This conservation also suggests that the MIRP is critical to the survival of the species and as such tolerates few, if any, substitutions in the primary amino acid sequence. Surprisingly, the first five residues of the MIRPs are nearly identical to the gonococcal protein I. Beyond residue five, no further homology is apparent. It is tempting to speculate that this sequence may encode some property such as function or cellular destination that is common to protein I and the MIRP. Alternatively, this sequence may be an evolutionary artifact, implying that the MIRP and molecules sharing an analogous sequence (such as the gonococcal protein I) may be descendents of some common progenitor protein. At this time, the significance of this conservation is not apparent.

Present speculation regarding the possible role of neisserial iron-regulated proteins in iron acquisition per se has been based on circumstantial evidence (8, 9, 31) and by analogy to other organisms. The studies reported here are the first to show that iron and a purified neisserial iron-regulated protein are stoichiometrically associated. The meningococcal and gonococcal MIRPs exhibit a pink color which coelutes with the MIRP from ion-exchange and gel-filtration columns, suggesting that a metal chromophore may associate with this protein. In quantitative terms, the MIRP contains ~0.5 mol iron per 1 mol protein. This finding is substantiated by the absence of iron associated with purified gonococcal protein I and the presence of 1.2 mol of iron per mol of the iron-binding protein transferrin, both analyzed using the same assay as a negative and positive control, respectively. Since saturated transferrin has two iron-binding sites, the iron determination performed in this study might represent an underestimation of MIRP-iron content. Moreover, the iron-MIRP complex must be quite stable since it is not dissociated in the presence of the detergent CTB.

Although the MIRP is both regulated by and associated with iron, this study did not attempt to show that MIRP exists in an iron-free form or that this molecule can bind iron (either directly or indirectly) from the environment. With regard to the latter, a previous report by Simonson et al. (10) analyzed the energy-independent binding of ^{55}Fe-citrate to membranes from iron-starved

meningococci. Analysis of the membranes after incubation with ^{55}Fe-citrate by SDS-PAGE showed that the iron isotope comigrated solely with a protein complex having an apparent molecular weight of 36,500. Based on relative molecular weights determined by SDS-PAGE, it is probable that the protein complex to which labeled iron was associated is identical to the protein that this study identifies as the meningococcal MIRP. This being the case, it is possible that the MIRP exists as an iron-binding component of meningococcal membranes and argues that the MIRP may play an integral role in iron-assimilation.

To date, the precise cellular role and pathologic significance of the MIRP has not been determined. This study describes the purification and biochemical characterization of the gonococcal and meningococcal MIRPs. The availability of this protein in pure and biologically relevant quantities will allow for several elucidating experiments to be performed and should significantly contribute to dissecting the pathobiologic function of this protein.

Summary

This report describes a method to purify the major iron-regulated protein (MIRP) expressed by *N. gonorrhoeae* and *N. meningitidis*. This purification procedure involves (*a*) maximal expression of the MIRP by growing the organisms on iron-limited media; (*b*) cellular disruption by sonication followed by centrifugal fractionation; (*c*) selective solubilization of the MIRP with the cationic detergent hexadecyltrimethylammonium bromide; (*d*) cation-exchange chromatography in the presence of this detergent; and (*e*) gel filtration chromatography. The MIRP purified by this technique migrates as a single band when analyzed by SDS-PAGE. The purified MIRP displayed an unusually basic isoelectric point, this value being >9.35. Further biochemical analysis revealed the highly conserved nature of this protein isolated from the two pathogenic species of the genus *Neisseria*. For example, the amino acid composition of the meningococcal and gonococcal MIRPs were nearly identical and amino terminal sequence analysis showed that both shared the identical primary sequence through residue 48. Surprisingly, the first five NH_2-terminal residues of the MIRPs exhibited homology with the first five residues of the gonococcal porin, protein I. Purified preparations of the MIRP exhibited a characteristic pink color reminiscent of the basic iron-binding protein lactoferrin. This observation coupled with the property of iron-regulation prompted us to analyze purified MIRP for iron-content. ~0.5 mol iron per 1 mol of MIRP was detected. This study is the first to show that iron is associated with the MIRP, a property that may implicate this protein as playing a direct role in neisserial iron assimilation. While the precise function of the MIRP is not known, the availability of this protein in pure and biologically relevent quantities will allow further studies to elucidate its pathobiologic function.

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