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Detection and Comparison of Specific Hemin Binding by *Porphyromonas gingivalis* and *Prevotella intermedia*

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A radioligand assay was designed to detect and compare specific hemin binding by the periodontal anaerobic black-pigmenting bacteria (BPB) *Porphyromonas gingivalis* and *Prevotella intermedia*. The assay included physiological concentrations of the hemin-binding protein rabbit serum albumin (RSA) to prevent self-aggregation and nonspecific interaction of hemin with cellular components. Under these conditions, heme-starved *P. intermedia* cells (two strains) expressed a single binding site species (4,100 to 4,600 sites/cell) with a dissociation constant (K_d) of 1.0×10^{-9} M. Heme-starved *P. gingivalis* cells (two strains) expressed two binding site species; the higher-affinity site (1,000 to 1,500 sites/cell) displayed a K_d of between 3.6×10^{-11} and 9.6×10^{-11} M, whereas the estimated K_d of the lower-affinity site (1.9×10^5 to 6.3×10^5 sites/cell) ranged between 2.6×10^{-7} and 6.5×10^{-8} M. Specific binding was greatly diminished in heme-replete cells of either BPB species and was not displayed by iron-replete *Escherichia coli* cells, which bound as much hemin in the absence of RSA as did *P. intermedia*. Hemin binding by BPB was reduced following treatment with protein-modifying agents (heat, pronase, and *N*-bromosuccinimide) and was blocked by protoporphyrin IX and hemoglobin but not by Congo red. Hemopexin also inhibited bacterial hemin binding. These findings indicate that both *P. gingivalis* and *P. intermedia* express heme-repressible proteinaceous hemin-binding sites with affinities intermediate between those of serum albumin and hemopexin. *P. gingivalis* exhibited a 10-fold-greater specific binding affinity and greater heme storage capacity than did *P. intermedia*, suggesting that the former would be ecologically advantaged with respect to heme acquisition.

The primary habitat of the anaerobic black-pigmenting bacteria (BPB) *Porphyromonas gingivalis* and *Prevotella intermedia* appears to be the human gingival crevice, and numerous studies have suggested that these microorganisms contribute to the etiology of various periodontal inflammatory conditions (for a review, see reference 24). The numbers and proportion of *P. gingivalis* generally increase with the development of adult periodontal disease (28, 29, 32), but the relationship of *P. intermedia* to periodontal disease is less clear (28, 32). *P. intermedia* may be isolated from healthy and mildly inflamed sites with greater frequency than *P. gingivalis* (28, 32), suggesting an ecological distinction between the two organisms.

Neither *P. gingivalis* nor *P. intermedia* is able to synthesize heme (iron protoporphyrin), which is an important growth factor for both species (11, 23). The major natural source of heme is probably the host organism (6), but the human circulatory system contains various heme-sequestering proteins, namely, serum albumin, hemopexin, and haptoglobin, which are also present in significant concentrations in gingival crevicular fluid (30). The heme released from damaged and senescent erythrocytes is potentially detrimental to mammalian cells because the combined lipophilic and oxidative nature of the molecule can damage membrane lipids and other macromolecules (26). Both human serum albumin (HSA) and hemopexin possess specific heme-binding sites and function to protect mammalian membranes by sequestering extracellular heme (26). The heme-hemopexin complex has a dissociation constant (K_d) of less than 10^{-12} M (14), and that of heme-albumin is around 10^{-8} M (1). Haptoglobin may also contribute to this protective effect by binding hemoglobin with extremely high affinity (K_d of $<10^{-15}$ M) (15). Furthermore, haptoglobin has been shown to protect mice from experimental peritoneal sepsis by withholding hemoglobin iron from infecting bacteria (10). If they are to assimilate host-derived heme, the BPB must wrest it from these scavenging proteins by proteolytic degradation and/or by expression of high-affinity binding sites.

In a complex microbial community such as exists in the gingival crevice, heme-requiring bacteria must also compete with one another (and probably other microorganisms) for available heme. In the relatively healthy gingival crevice, any heme present should be tightly bound to specific host proteins, limiting its availability and possibly influencing the microbial ecosystem by selectively facilitating growth of those organisms able to acquire the bound heme. The present investigation sought to develop a radioligand assay with which to detect, measure, and compare specific hemin binding by *P. gingivalis* and *P. intermedia.*

MATERIALS AND METHODS

Bacterial strains and culture conditions. *P. gingivalis* ATCC 33277 and WT40 were kindly provided by G. R. Germaine (University of Minnesota). *P. intermedia* ATCC 25611 and *Escherichia coli* ATCC 10798 (K-12) were obtained from the American Type Culture Collection (Rockville, Md.). *P. intermedia* MCG1 was isolated from a patient suffering from mild gingivitis by plating a subgingival plaque sample directly onto tryptic soy broth (TSB) agar (Difco Laboratories, Detroit, Mich.) supplemented with hemin (5 μg/ml), menadione (0.5 μg/ml), and sheep whole blood (5% [vol/vol]) (sTSB agar). Plates were incubated anaerobically for 7 days, and a black-pigmented colony was isolated by subculturing onto sTSB agar. The identities of bacterial strains were confirmed by the An-Ident bacterial identification method (API Analytab Products, Plainview, N.Y.). BPB strains were maintained by weekly subculture on sTSB agar, and *E. coli* was maintained on Luria-Bertani agar (Difco).

To prepare bacterial cells for hemin binding assays, all culture vessels were presoaked in chromic-sulfuric acid and thoroughly rinsed with deionized water. Heme-starved BPB cells were prepared by growth at 37°C in prereduced TSB supplemented with menadione (TSB-M), whereas heme-replete cells were grown in TSB-M supplemented with hemin (5 μg/ml). Cultures were incubated until the

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cells had entered the stationary phase of growth (determined turbidometrically), generally between 24 and 48 h. Prior to harvesting, a sample of each culture was (i) streaked onto sTSB to check the purity and (ii) inoculated into TSB-M to determine the nutritional status of the cells with respect to heme requirement; fully heme-starved bacteria grew poorly or not at all following reinoculation into TSB-M. Cultures were removed from the anaerobic environment, and the cells were killed by addition of sodium azide (to 3 μM). Ten minutes later, the cells were washed by three cycles of centrifugation and resuspension in the appropriate buffer (see below).

Biochemicals. HSA, rabbit serum albumin (RSA), human haptoglobin, 3,3′,5,5′-tetramethylbenzidene (TMBZ), phenylmethanesulfonyl fluoride, 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine), DL-cysteine, protoporphyrin IX, and hemin were purchased from the Sigma Chemical Co. (St. Louis, Mo.). *N*-Bromosuccinimide (NBS) was from ICN Biomedicals Inc. (Aurora, Ohio). Tosyllysyl chloromethyl ketone (TLCK) was from Boehringer Mannheim Corp. (Indianapolis, Ind.). [^{14}C]hemin (105 Ci/mol) was from Leeds Porphyrins (Leeds, United Kingdom).

Human apohemopexin was purified from plasma by a combination of hemin-agarose (Sigma) affinity chromatography and ion-exchange chromatography (31). The preparation was assessed for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (17); one half of the gel was stained with Coomassie brilliant blue G250 (19), and the other half, containing identical lanes, was analyzed by immunoblotting using rabbit antiserum to human hemopexin (Dako Corporation, Carpinteria, Calif.) as the primary antibody and an alkaline phosphatase-conjugated secondary antibody detection system (Bio-Rad Laboratories, Hercules, Calif.). The hemopexin preparation was deemed 94% pure by densitometric analysis of the Coomassie brilliant blue-stained gel.

Determination of heme and iron content of bacteria. Bacteria were thoroughly washed and resuspended in deionized water. The suspensions were desiccated to equilibrium in preweighed, acid-washed ceramic crucibles, and the crucibles were reweighed to determine the dry weight of the bacteria. Preparations were ashed by heating to 800°C for 4 h, and after cooling, the total iron content of the ash was measured by reaction with ferrozine (8). The endogenous heme concentrations of washed bacterial suspensions (of known dry weight) were determined by serial dilution in distilled water followed by drying in a vacuum centrifuge. The dried cells were resuspended in glacial acetic acid and boiled for 15 min, and the acetic acid was removed by evaporation. TMBZ reagent and hydrogen peroxide (3%) were added to the preparations, which were then mixed and left at room temperature for 1 h before centrifugation and spectrophotometric measurement of the supernatants (13). Heme equivalents were determined by comparison to a calibration series prepared with hemin.

[^{14}C]hemin binding assay. The capacity of bacteria to bind free (i.e., uncomplexed) hemin was determined by incubating washed bacteria in Tris-buffered saline (TBS; 10 mM Tris buffer [pH 7.1 at 37°C], 130 mM NaCl) containing TLCK (1 mM) and [^{14}C]hemin (0.1 μM). Following incubation for 1 h at 37°C, the bacteria were recovered by centrifugation (14,000 × *g* for 2 min) and washed three times in TBS containing 0.1% (vol/vol) polyoxyethylene-sorbitan monolaurate (Tween 20) (TBS-T). The cell-bound radiolabel was then determined by liquid scintillation spectroscopy and subtraction of background counts (see below). The effects of various agents on [^{14}C]hemin binding were assessed by inclusion of (i) nonradioactive hemin (100 μM), (ii) HSA (20 mg/ml), or (iii) RSA (20 mg/ml). The assay was also used to determine the capacity of previously heated (80°C for 15 min followed by thorough washing) bacteria to bind free [^{14}C]hemin.

For reasons explained below, provision of uncomplexed [^{14}C]hemin to bacteria was deemed an unsuitable approach to determining specific hemin binding, and the assay was modified in the following manner. Stock suspensions of washed bacteria were preincubated with TLCK (10 mM) for 15 min at 37°C in TBS. The cell concentration of the stock suspension used in each assay was determined with a Petroff-Hausser bacterial counting chamber (Hausser Scientific Partnership, Horsham, Pa.). Concurrently, two series of tubes containing RSA (20 mg/ml) and a range of concentrations of [^{14}C]hemin were prepared. One series was further supplemented with unlabelled hemin (100 μM), and all tubes were preincubated for 15 min. The assay was initiated by addition of the bacteria to approximately 5×10^9 cells/ml, and incubation, with end-over-end rotation (15 rpm), proceeded for 15 min (unless otherwise indicated). Assay tubes were then centrifuged (14,000 × *g* for 2 min), and the cells were washed three times in TBS-T as described above. The pelleted cells were carefully drained, and the associated radiolabel was measured by liquid scintillation spectroscopy in a model LS3801 counter (Beckman Instruments, Fullerton, Calif.). Background counts were determined from assay tubes containing [^{14}C]hemin and all other components except the bacteria and did not exceed blank counts (no added radiolabel) by more than 50%. Counts of nonradioactive hemin samples were not significantly higher than the counter background, indicating no chemiluminescent interference. Specific hemin binding was calculated by subtracting the concentration of [^{14}C]hemin bound in the presence of excess unlabelled hemin (nonspecific binding) from that bound in the absence of the supplemental hemin (total hemin binding) (5). Data were transformed (22) and analyzed by either linear or nonlinear regression (Enzfitter version 1.05; Elsevier-Biosoft, Cambridge, United Kingdom) to determine the apparent dissociation constants and concentrations of the binding sites. The dissociation constant for each site was calculated from the apparent dissociation constant by stoichiometric adjustment for the significant hemin-binding capacity of the RSA (18).

TABLE 1. Comparison of total iron and heme contained in heme-starved and heme-replete *P. gingivalis* and *P. intermedia* and in iron-replete *E. coli* cells[a]

Strain	Growth conditions	Iron (μmol/g [dry wt])	Heme (nmol/g [dry wt])
P. gingivalis			
ATCC 33277	Heme replete	0.94 ± 0.12	513 ± 9
	Heme starved	1.71 ± 0.73	2.50 ± 0.39
	PPIX[b] replete	0.87 ± 0.15	1.48 ± 0.39
WT40	Heme replete	0.21 ± 0.04	434 ± 11
	Heme starved	2.32 ± 0.13	3.24 ± 0.12
P. intermedia			
ATCC 25611	Heme replete	2.95 ± 0.41	13.74 ± 1.11
	Heme starved	3.95 ± 0.46	<1.0
	PPIX replete	3.85 ± 0.35	5.09 ± 2.32
MCG1	Heme replete	1.41 ± 0.13	14.2 ± 1.65
	Heme starved	1.58 ± 0.16	<1.0
E. coli ATCC 10798	Iron replete[c]	2.17 ± 0.16	3.98 ± 1.45

[a] Total iron was determined by the ferrozine method, and heme was determined by the TMBZ method. Values are means ± standard deviations of triplicate assays.
[b] PPIX, protoporphyrin IX.
[c] Culture was not supplied with hemin.

Effects of heme analogs and heme-sequestering proteins on hemin binding by bacteria. Various agents were assessed for the capacity to competitively block [^{14}C]hemin binding by heme-starved bacteria. Bacteria were prepared as described above and added to assay mixtures containing RSA (20 mg/ml), [^{14}C]hemin (0.1 μM), and the potential competitor at a 10- or 100-fold molar excess. The effect of apohemopexin was similarly determined by including the protein at a molar concentration approximately twice that of the radioligand. The capacity of human haptoglobin to prevent the competitive blocking effect of human hemoglobin was determined by adding haptoglobin at approximately twice the molar concentration of the hemoglobin. Unlabelled hemin was used as a positive control for all inhibition experiments to determine the extent of specific hemin binding.

Heat, enzyme, and chemical modification of bacterial heme-binding sites. To investigate the biochemical nature of the heme-binding sites, the effects of various bacterial pretreatments on [^{14}C]hemin binding were assessed. For experiments involving heated bacteria, concentrated cell suspensions (0.5 ml) were incubated for 15 min at 80°C in a water bath followed by thorough washing in TBS. Selective amino acid oxidation of proteins (27) was achieved by incubating washed cells in TBS (control) or in TBS containing NBS (1 mM) at room temperature for 60 min followed by thorough washing in TBS. The effects of proteolytic enzyme treatment were determined by incubating washed bacteria with either pronase (1 mg/ml) or trypsin (1 mg/ml) or without enzymes (control) for 1 h at 37°C with continuous mixing. Incubation was terminated by addition of phenylmethylsulfonyl fluoride (1 mM) and TLCK (1 mM) with cooling on ice for 10 min followed by thorough washing in TBS. Treated and untreated bacterial suspensions were adjusted to the same cell concentration (±5%), and total hemin binding was determined by the [^{14}C]hemin binding assay using one radioligand concentration (0.1 μM) in the presence of RSA (20 mg/ml) and TLCK (1 mM).

RESULTS

Endogenous heme and iron content of bacteria. Table 1 records the amounts of TMBZ-reactive material (i.e., heme) and the total cellular iron present in *P. gingivalis*, *P. intermedia*, and *E. coli* cells following growth under different conditions of heme availability. The measured heme content of cells grown under heme-deficient conditions compared to their heme-replete counterparts and the poor or absent growth on subsequent subculture confirmed that the cells were relatively heme deficient. Iron was not removed from the base medium before inoculation because the menadione-supplemented medium did not maintain the growth of BPB, but, unexpectedly, the heme-starved cells contained slightly greater amounts of total iron

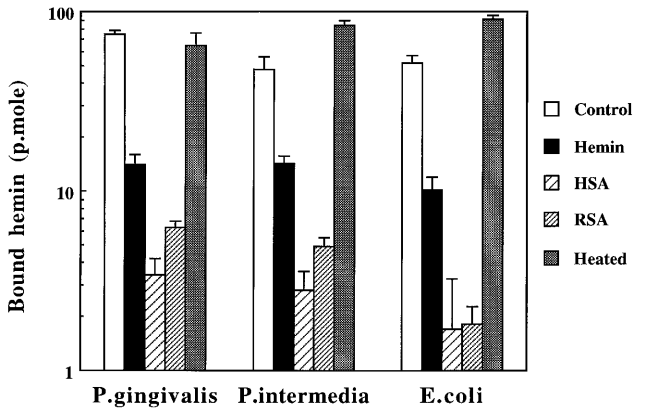

FIG. 1. Effects of heat, serum albumin, and excess hemin on binding of [^{14}C]hemin by *P. gingivalis* ATCC 33277, *P. intermedia* MCG1, and *E. coli* ATCC 10798. Washed, TLCK-treated bacteria (5×10^9/ml) were incubated for 15 min at 37°C in Tris-buffered saline with [^{14}C]hemin (0.1 μM) and (i) no supplements (control), (ii) nonradioactive hemin (100 μM), (iii) HSA (20 mg/ml), and (iv) RSA (20 mg/ml). Heated (15 min at 80°C) bacteria were incubated without supplements. *P. intermedia* and *P. gingivalis* cells were heme starved, and *E. coli* was iron replete. Data are means and standard deviations of triplicate assays.

than did the heme-replete cells. Addition of protoporphyrin IX did not restore cellular heme levels, but protoporphyrin IX-supplemented *P. intermedia* cells contained more heme than did porphyrin-deficient cells. Heme-replete *P. gingivalis* cells accumulated comparatively large amounts of heme (at least 30 times greater than the amounts accumulated by *P. intermedia*), and probably as a consequence, we encountered difficulty depleting the cellular heme concentrations to the same degree as for *P. intermedia* cells. Unless otherwise indicated, all subsequent experiments involved heme-starved BPB cells and iron-replete *E. coli* cells.

Effects of serum albumin on hemin binding by bacteria. The capacities of metabolically inactive *P. intermedia*, *P. gingivalis*, and *E. coli* cells to bind uncomplexed [^{14}C]hemin and the effects of nonradiolabelled hemin and serum albumins on [^{14}C]hemin binding are compared in Fig. 1. The *E. coli* strain was included because it is a gram-negative bacterium without a specific requirement for heme and therefore acts to assess whether hemin binding is a unique property of the BPB or a general phenomenon applying to other gram-negative bacteria. *P. gingivalis* bound more free hemin than did either *P. intermedia* or *E. coli*, but *E. coli* bound slightly more than did *P. intermedia* (Fig. 1). Preheated cells of *P. intermedia* and *E. coli* bound more free hemin than did their respective unheated controls, but heating had little effect on binding by *P. gingivalis*. For all strains, [^{14}C]hemin binding was partially blocked (70 to 81%) by excess nonradioactive hemin (100 μM) but almost completely inhibited (≥90%) by either RSA or HSA (Fig. 1).

Specific binding of [^{14}C]hemin by bacteria. Initial studies indicated that a conventional approach to determination of specific hemin binding by subtracting nonspecific binding from total binding (5) was not feasible for assays using the uncomplexed ligand. The lipophilic nature of the hemin molecule probably causes it to adhere to most biological surfaces when provided in free form. This nonspecific effect produced such high values for total binding that, with the limited aqueous solubility of the hemin, it was not possible to achieve a great enough concentration of unlabelled ligand to determine specific binding (data not shown). To overcome such difficulties, RSA was included in the assay at a molar concentration exceeding that of the total hemin, as a hemin-binding agent to prevent both nonspecific binding and self-aggregation (20) of the hemin. The inclusion of TLCK in the assay mixture reduced proteolytic digestion of RSA to less than 5% of total protein over a 2-h incubation period when exposed to either *P. gingivalis* or *P. intermedia* cells, as determined by generation of trichloroacetic acid-soluble UV-absorbing (A_{280}) material (data not shown). Furthermore, by comparison to the migration of untreated RSA, sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that the RSA remained intact throughout this incubation (data not shown). RSA also eliminated hemin binding to the assay vessels and was used in preference to HSA in saturation binding assays because the dissociation constant is more precisely known (18). Although total [^{14}C]hemin binding in the presence of RSA approximated specific binding, initial experiments revealed that the RSA-hemin complex also bound to the bacteria to some extent. This binding effect was not specific for the complex, as it was reduced by increasing the RSA concentration (data not shown), and it was therefore necessary to measure nonspecific binding by including a radioligand concentration series containing additional excess nonradioactive ligand.

The modified assay, which distinguished total from nonspecific hemin binding in the presence of RSA, produced a saturating plot for specific binding by *P. intermedia* (Fig. 2A). Transformation of the data by the method of Rosenthal (22) produced a linear plot indicating the presence of a single binding site species (Fig. 2B). The concentration of binding sites was determined by extrapolation, and the dissociation constant was calculated from the slope of the plot (5). Two tested strains of *P. intermedia* exhibited essentially the same response (Table 2). Examination of *P. gingivalis* by the same technique failed to produce a clearly saturating plot for specific binding (Fig. 2C), but transformation of the data produced a nonlinear plot indicating more than one binding site (Fig. 2D). Determination of the parameters governing the higher-affinity site (line 1 in Fig. 2D and site 1 in Table 2) by nonlinear regression indicated an affinity 10-fold greater than that displayed by the *P. intermedia* strains tested, though with about one-fourth the concentration of binding sites (Table 2). The second site (assuming two sites) was of substantially lower affinity than that of *P. intermedia* but was expressed at a relatively high concentration (line 2 in Fig. 2D and site 2 in Table 2). Again, two tested strains of *P. gingivalis* produced essentially the same result (Table 2).

Specific hemin binding was considerably diminished in heme-replete cells of both *P. intermedia* and *P. gingivalis*, and saturable binding was not convincingly demonstrated within the same ligand concentration range as that used for heme-starved cells (data not shown). Transformation of the binding data produced plots which could not be satisfactorily resolved by regression analysis (Fig. 2B and D). Specific hemin binding could not be detected at all with iron-replete *E. coli* cells (Fig. 2E), and the transformed data clustered around the origin (Fig. 2F).

The time required for specific hemin binding to reach equilibrium was determined with two series of tubes (i.e., with and without excess unlabelled hemin) prepared as described above but with a single concentration of radiolabelled hemin throughout. Following addition of bacteria, the tubes were incubated and triplicate tubes from both series were removed at intervals and processed as described above to measure bound hemin. Specific hemin binding by both *P. intermedia* and *P. gingivalis* reached equilibrium in 5 to 10 min, but after 30 min, binding by *P. intermedia* began to deteriorate significantly whereas that of *P. gingivalis* was relatively stable (data not shown). Thus, most of our binding studies used a 15-min in-

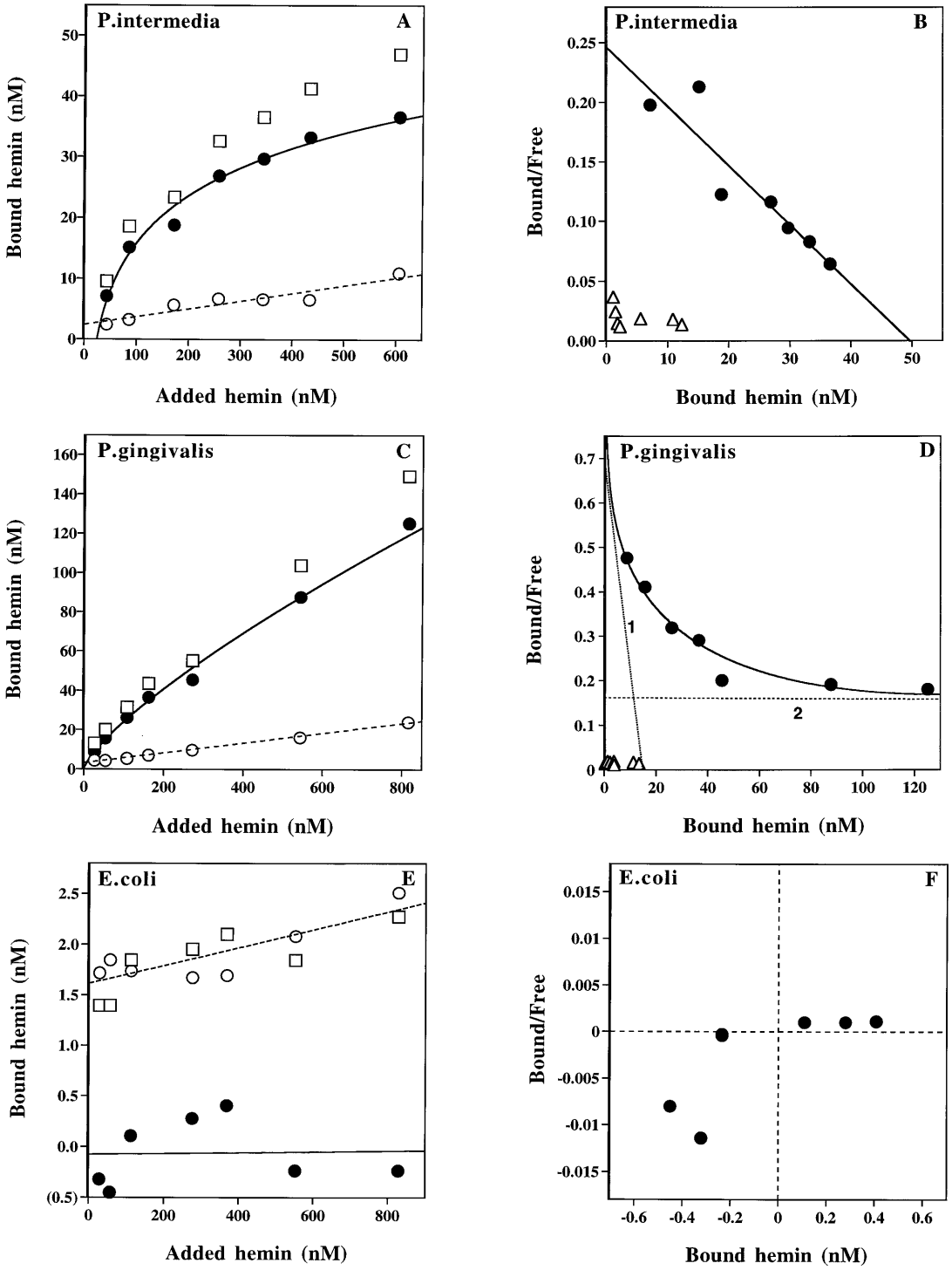

FIG. 2. Detection and measurement of specific hemin binding by *P. intermedia* MCG1, *P. gingivalis* ATCC 33277, and *E. coli* ATCC 10798. Washed bacteria (5×10^9/ml) were incubated with a range of concentrations of [^{14}C]hemin in the presence of RSA (20 mg/ml) with and without nonradioactive hemin (100 μM). (A, C, and E) Specific binding (●) by heme-starved bacteria, calculated by subtracting nonspecific (○) from total (□) binding; (B, D, and F) transformation and comparison of specific binding data for heme-starved (●) and heme-replete (△) bacteria. Linear components (lines 1 and 2) of the *P. gingivalis* curve (D) were resolved by nonlinear regression analysis.

cubation period for both organisms. The effect of pH on hemin binding was determined by using buffers adjusted over a pH range from 6.5 to 8.5 and a single concentration of radiolabelled hemin (with and without excess unlabelled hemin). For both BPB species, maximal specific binding occurred between pH 6.5 and 7.5, but at pH 8.5, binding by both species was less than 60% of maximal binding (data not shown).

Characterization of bacterial hemin-binding sites. The ligand specificity of the detected binding sites was assessed by incubating bacteria with [^{14}C]hemin, RSA, and a molar excess

TABLE 2. Summary of hemin-binding parameters for isolates of *P. gingivalis* and *P. intermedia*

Strain	Binding site designation	No. of binding sites/cell (± SE)	K_d[a] (M) (± SE)
P. gingivalis			
ATCC 33277	1	1,492 ± 601	$3.6 \times 10^{-11} \pm 4.6 \times 10^{-11}$
	2	$6.3 \times 10^5 \pm 9.2 \times 10^5$	$6.5 \times 10^{-8} \pm 9.6 \times 10^{-8}$
WT40	1	1,042 ± 50	$9.6 \times 10^{-11} \pm 5.6 \times 10^{-12}$
	2	$1.9 \times 10^5 \pm 2.3 \times 10^5$	$2.6 \times 10^{-7} \pm 3.1 \times 10^{-7}$
P. intermedia			
ATCC 25611	1	4,191 ± 602	$1.0 \times 10^{-9} \pm 2.6 \times 10^{-10}$
MCG1	1	4,560 ± 203	$9.4 \times 10^{-10} \pm 8.5 \times 10^{-11}$

[a] Adjusted to account for the hemin-binding affinity of RSA ($K_d = 5 \times 10^{-7}$ M) (18).

of one of several potentially competing compounds. Figure 3 shows that for both *P. intermedia* and *P. gingivalis*, protoporphyrin IX and hemin were effective in preventing radioligand binding whereas the heme analog Congo red was ineffective. Cytochrome *c*, which contains a covalently bound heme group, was also unable to prevent hemin binding by either BPB species. Hemoglobin (at a relatively low concentration) prevented radioligand binding by *P. intermedia* but was only partially effective against *P. gingivalis* (Fig. 3). The haptoglobin-hemoglobin complex also blocked hemin binding by *P. intermedia*, but this effect was less clear against *P. gingivalis* (Fig. 3). Hemopexin (added as the apoprotein) completely prevented specific binding of [^{14}C]hemin by both BPB species (Fig. 3). These experiments were repeated in buffer containing cysteine (1 mM) to determine the effect of reducing conditions. The results were similar to those in Fig. 3, but the cysteine caused a slight (≈10%) overall reduction in total hemin binding (data not shown).

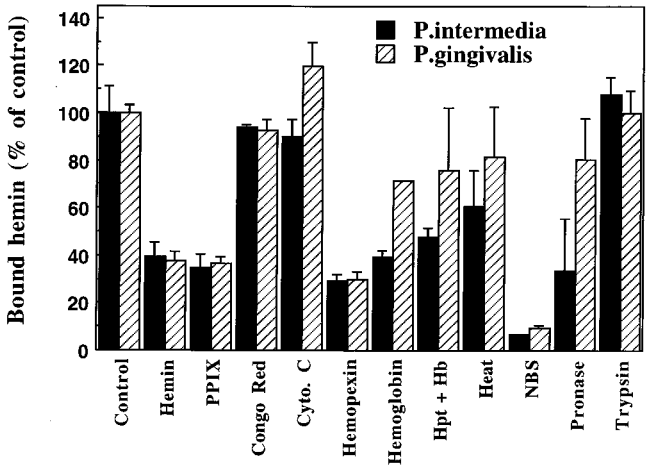

FIG. 3. Effects of heme analogs, heme-sequestering proteins, and bacterial pretreatments on total hemin binding by heme-starved *P. intermedia* MCG1 and *P. gingivalis* ATCC 33277 cells. For all assays, washed bacteria (5×10^9/ml) were incubated for 15 min at 37°C with RSA (20 mg/ml), TLCK (1 mM), and [^{14}C]hemin (0.1 μM) before washing and determination of cell-bound radiolabel. The effects of heme analogs and heme-sequestering proteins were examined by including hemin (100 μM), protoporphyrin IX (PPIX; 100 μM), Congo red (100 μM), cytochrome *c* (Cyto. C; 100 μM), apohemopexin (14 μM), hemoglobin (10 μM), or hemoglobin (10 μM) and haptoglobin (20 μM) combined (Hpt + Hb). Cell pretreatments included heating to 80°C for 15 min (heat), incubation with NBS (2 mM) for 1 h at 25°C, and exposure to either trypsin or pronase (1 mg/ml) for 1 h at 37°C. All pretreatments were followed by thorough washing before addition of bacteria to assay tubes. Bound hemin is expressed as a percentage of the appropriate control (see Materials and Methods), but the effect of excess unlabelled hemin (100 μM) on untreated cells is shown to indicate the extent of nonspecific binding. Data are means and standard deviations of triplicate assays.

Essentially the same assay was used in studies undertaken to ascertain the biochemical nature of the bacterial hemin-binding sites. Prior treatment of bacteria with the protein-modifying reagent NBS effectively resulted in complete loss of total hemin-binding activity for both *P. intermedia* and *P. gingivalis*, whereas heating the bacteria only partially abrogated binding (Fig. 3). Preexposure of bacteria to pronase caused complete loss of the specific binding activity of *P. intermedia* but only partial damage to the *P. gingivalis* binding sites. Trypsin, on the other hand, was ineffective against both organisms (Fig. 3).

DISCUSSION

A number of pathogenic bacteria are able to use heme as either an iron or an iron-porphyrin source, and specialized mechanisms for iron and heme uptake are generally considered virulence factors (for a review, see reference 21). In any ecological system, those organisms expressing the most proficient mechanism for acquiring a specific nutrient or growth factor should be ecologically advantaged under conditions of limited growth factor availability. By measuring the dissociation constants of the binding sites, this study found that *P. gingivalis* bound hemin with approximately 10-fold-greater affinity than did *P. intermedia*, though it expressed only a quarter of the number of high-affinity sites. Furthermore, *P. gingivalis* expressed an additional specific site of lower affinity, and as this species exhibited a considerably greater capacity to accumulate heme, the function of the second site may relate more to heme storage than to direct acquisition of exogenous heme. Overall, *P. gingivalis* appeared to be the more avid heme scavenger, and competition for heme is therefore probably not the critical factor facilitating the relative prevalence of *P. intermedia* in the heme-restrictive environment of healthy and mildly inflamed gingival sites.

Although clearly greater than that of serum albumin, the affinities of the specific hemin-binding site of *P. intermedia* and the higher-affinity site of *P. gingivalis* were considerably less than that of hemopexin, implying that these bacteria require auxiliary mechanisms to acquire heme in the presence of this plasma/gingival crevicular fluid protein. The hemin-withholding effect of hemopexin was also confirmed experimentally. In contrast, haptoglobin was unable to prevent the hemin-blocking effect of hemoglobin against *P. intermedia*, suggesting that this organism can acquire the haptoglobin-complexed heme. This effect was less decisive in experiments with *P. gingivalis* because of the limited binding inhibition effect of hemoglobin, which in turn was probably due to the high concentration of the lower-affinity hemin-binding site of the bacterium. However, the K_d of the higher-affinity site suggests that *P. gingivalis* could also acquire heme directly from the haptoglobin-hemoglobin complex. Indeed, Bramanti and Holt (2) reported that the growth of *P. gingivalis* in a bacteriological medium is unhindered by the presence of either haptoglobin or hemopexin (with their respective ligands as the heme source), whereas *E. coli* is unable to grow under these conditions but proliferates in the absence of the heme-sequestering proteins.

Proteolytic degradation seems the most likely mechanism by which the BPB could release heme from hemopexin. In fact, hemopexin is highly susceptible to proteolytic cleavage by *P. gingivalis*, whereas *P. intermedia* is less effective in this respect (6, 16). Serum albumin is of limited susceptibility to degradation by either species (6, 16), but the current investigation,

which used protease inhibitors throughout, indicated that albumin degradation is not necessary for heme binding by the BPB. A postulated mechanism for the uptake of heme by *P. gingivalis* would therefore involve degradation (possibly specific) of heme-hemopexin by bacterial proteases and binding of the released heme by the higher-affinity specific site, in the presence of intact albumin.

Unexpectedly, heme-starved cells of both BPB species contained at least as much total iron as did the corresponding heme-replete cells, implying the expression of an iron acquisition mechanism distinct from, but perhaps regulated by, the heme uptake system. The increased accumulation of iron was presumably stimulated to compensate in some way for the deficiency of heme. Bramanti and Holt (2) have shown that *P. gingivalis* can grow comfortably when high concentrations of inorganic iron replace hemin. Whether the BPB could acquire sufficient amounts of inorganic iron to maintain metabolic processes under natural circumstances, however, remains to be determined.

Previous reports have suggested that binding of uncomplexed hemin by *P. gingivalis* is mediated by the lipid A component of the lipopolysaccharide (LPS) constituent of the outer membrane (12), but as hemin is a notably lipophilic molecule (26), this effect is perhaps to be expected. We are unaware of evidence demonstrating that LPS-mediated hemin binding is unique to the BPB, and the current study found that iron-replete *E. coli*, which does not have a requirement for exogenous heme, bound as much uncomplexed hemin as did *P. intermedia*. This nonspecific effect was inhibited by albumin, implying that when provided in the free form, most of the cell-associated hemin was bound with relatively low affinity (i.e., lower than that of albumin). We suspect that most gram-negative bacteria will exhibit a similar nonspecific hemin-binding behavior, and in our opinion, LPS-mediated hemin binding is probably not biologically relevant because of the low affinity of the interaction and the abundant presence of host plasma proteins which function to counter the lipophilic disposition of the heme (26). In the current study, specific binding was measured in the presence of high concentrations of albumin to prevent both the lipophilic and self-aggregating effects of the hemin. Nonspecific LPS-mediated hemin binding is probably responsible for the paradoxical observation that heme-replete *P. gingivalis* cells bind more hemin than do heme-starved cells, first reported by Carman et al. (7). Recently, Cutler et al. (9) demonstrated that heme availability influences the LPS composition of *P. gingivalis* and that the LPS extracted from heme-replete cells binds more hemin than does that from heme-starved cells. Whereas this finding provides an explanation for the observations of Carman et al. (7), it does not imply that hemin binding is a biological function of LPS.

Opposing the view that heme binding is mediated by LPS, several studies (including the present one) indicate that heme starvation stimulates expression of hemin-binding surface proteins by *P. gingivalis* (3, 4, 25). Bramanti and Holt (3) identified a hemin-repressible protein in the outer membrane of heme-starved *P. gingivalis* cells, which was subsequently shown to bind hemin (4). In the current investigation, the hemin-binding site of *P. intermedia* was inactivated by both pronase and NBS, implying an essential protein component. Both *P. gingivalis* sites were inactivated by NBS, but pronase caused only a limited reduction in specific binding, possibly because only one of the two sites was sensitive. As the putative primary binding structure, the higher-affinity site is probably surface exposed and accessible to pronase whereas the lower-affinity site, if it functions to store heme, may be sequestered within the outer membrane.

In conclusion, our study found that *P. gingivalis* displayed a 10-fold-greater specific affinity for hemin than did *P. intermedia*. Specific hemin binding was maximally expressed by heme-starved cells, and the binding sites of both organisms appeared to be protein in nature.

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