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Partial Characterization and Staphylocidal Activity of Thrombin-Induced Platelet Microbicidal Protein

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Thrombin-induced platelet microbicidal protein (PMP) is considered to play an important role in preventing streptococcal endocarditis. However, the structural features and functions of PMPs have not been well characterized, and their antibacterial spectra against other common endocarditis pathogens, such as the staphylococci, are not known. Thrombin stimulation of washed rabbit platelets (10^8/ml) yielded a PMP-rich preparation with a specific activity of ~25 U/mg of protein as determined by *Bacillus subtilis* bioassay. Twenty-eight clinical and laboratory *Staphylococcus aureus* isolates, exposed to a standardized PMP preparation (100 U/ml for 2 h at 37°C), exhibited a Poisson-distributed heterogeneity to the bactericidal action of PMP, with approximately one-third designated as PMP resistant. Gel filtration chromatography (Sephadex G-50) identified the bioactive moiety within PMP preparations to be in the major protein elution peak; sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) presumptively identified PMP as a low-molecular-weight (MW) (8,500) protein present only in such bioactive protein peaks. Both the bioactivity of PMP preparations and the low-MW protein band were removable by specific anionic membranes (e.g., cellulose-acetate/nitrate), as well as by a variety of anionic resins, further corroborating the suspected cationic charge of PMP. In addition, both PMP bioactivity and the low-MW protein band were recoverable by 1.5 M NaCl elution of the anionic membrane filters post-PMP adsorptive removal. Adsorption of bioactive PMP preparations by highly PMP-susceptible *B. subtilis* (10^8 CFU/ml, 30 min) resulted in a near-complete loss of residual bioactivity; in contrast, adsorption of bioactive PMP preparations with less PMP-susceptible *S. aureus* strains failed to reduce bioactivity. Significant lysozyme contamination of PMP-rich preparations was ruled out by determination of differences between bioactive PMP preparations and exogenous lysozyme as regards (i) relative heat stabilities; (ii) differential bactericidal activity versus *B. subtilis* and *Micrococcus luteus*; and (iii) SDS-PAGE protein profiles. These data show that the bioactive PMP protein moiety is of low MW, is heat stable, is probably cationic (similar to leukocyte-derived defensins), and possesses potent bactericidal activity against a significant percentage of *S. aureus* isolates.

Bacterial-platelet (PLT) interactions represent a critical first step in the induction of infective endocarditis (IE [9, 10]). The ability of an organism to induce IE appears to involve both an ability to adhere to and aggregate PLTs on the one hand (10, 21) and evasion of the antibacterial capacity of PLTs on the other hand (4). Although PLTs may occasionally internalize bacteria via phagocytosis, intracellular killing does not occur (4); rather, the primary mechanism of PLT antibacterial host defense is generally considered to involve α-granule-derived bactericidal proteins (5, 6). During generalized clotting, rabbit and human PLTs release bactericidal substances which possess activity predominantly against gram-positive organisms (6, 14, 23). Similarly, following specific exposure to thrombin, a release of α-granule-derived PLT microbicidal protein(s) (PMPs) with bactericidal activity against classical IE pathogens such as viridans streptococci has been documented (5). These findings have led to the notion that organisms intrinsically resistant to PMP(s) would manifest a selective survival advantage during PLT-bacteria interactions at the cardiac vegetation surface. Such thrombin-induced antibacterial proteins have also been termed thrombodefensins (5); however, their exact relationship to other defensin molecules, such as the leukocyte defensins (12), has not been defined.

Thrombin-induced PMP(s) remain poorly characterized, and their structure, function, and microbicidal pathways have not been fully determined. In the present study, we describe the preparation, partial purification, and initial characterization of thrombin-induced PMP. In addition, we utilized *Staphylococcus aureus*, the most common etiologic agent of intravascular infections (19), to probe the functional microbicidal features of PMP.

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MATERIALS AND METHODS

Preparation of thrombin-induced PMP. Central ear arterial blood from healthy New Zealand White rabbits was freshly collected into citrated, siliconized tubes. Low-speed (75 × *g*) centrifugation of blood samples produced a loose erythrocyte pellet and an upper, PLT-rich plasma supernatant. Collection of the upper two-thirds of the PLT-rich plasma supernatant routinely yielded PLTs having <1% leukocyte

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contamination. The PLT-rich plasma fraction was collected into citrated, siliconized tubes and centrifuged for 10 min at 2,000 × *g* to pellet the PLTs. The resulting PLT pellet was washed twice in Tyrode salts solution (0.08 mM NaCl, 3.8 mM K_2HPO_4, 4.0 mM Na_2HPO_4, 2.8 mM glucose, 16.6 mM citric acid, 34 mM sodium citrate [pH 6.8]; Sigma Chemical Co., St. Louis, Mo.) and then resuspended in Eagle's minimal essential medium (MEM) to a concentration of $\sim 10^8$ PLTs per ml as determined by Coulter counting (Coulter Instruments, Hialeah, Fla.) and/or spectrophotometry (optical density, λ = 600 nm; Spectronic 401; Milton Roy Analytical Division, Rochester, N.Y.). PMP-rich preparations were produced from washed PLTs by thrombin stimulation of $\sim 10^8$ washed PLTs per ml in MEM (1 U of thrombin [Sigma Chemical Co.] with 12.5 μl of 0.2 M $CaCl_2$ per ml of washed PLT suspension; 20 min, 37°C [5]). Following this thrombin-induced PLT α-granule secretion, residual PLT material was removed by centrifugation (2,000 × *g*, 10 min), and the PMP-rich supernatant was recovered. PMP preparations were stored at −20°C and used for subsequent studies within 30 days.

Determination of PMP bactericidal activity. The total protein content of PMP preparations was determined spectrophotometrically (18), and the PMP bactericidal activity was determined by techniques modified from those of Donaldson and Tew (6). Briefly, bioactivity assays were performed with *Bacillus subtilis* (ATCC 6633), an indicator organism highly sensitive to the bactericidal action of PLT-derived PMP (5). *B. subtilis* was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) at 37°C (14 h); organisms were harvested by centrifugation, washed twice in normal saline, and resuspended in phosphate-buffered saline (PBS; pH 7.2) prior to use. All PMP bioactivity assays were performed in low-protein-binding microtiter plates (Corning Glass Works, Corning, N.Y.). A *B. subtilis* inoculum of 10^4 CFU/ml was added to microtiter wells containing a range of dilutions of the PMP-rich preparation to achieve a final inoculum of 10^3 CFU/ml per well and a final range of PMP dilutions of 1:1 to 1:1,024 (final well volume = 200 μl); one well contained *B. subtilis* in MEM alone as a positive growth control. The microtiter plates were then incubated in ambient CO_2 (37°C). At 0, 30, 60, and 120 min of incubation, 20-μl aliquots were removed from each well, diluted into PBS containing 0.01% sodium polyanetholsulfonate (SPS; to inhibit further PMP-induced bacterial killing), and quantitatively cultured onto 6.6% sheep blood agar. Killing curves were then constructed comparing percent *B. subtilis* survival over time. In parallel, the optical densities of each combination of PMP plus *B. subtilis* were determined spectrophotometrically (λ = 600 nm) to monitor bacterial cell lysis over time, in order to compare the time-dependent bactericidal versus bacteriolytic actions of PMP. PMP bioactivity was defined as the inverse of the highest PMP dilution (in units per milliliter) which retained ≥95% lethality versus *B. subtilis* (6). The PMP specific activity was then defined and quantified as PMP bioactivity units per milligram of protein. Thrombin (1 U/ml) in PBS or MEM and supernatants from washed PLTs not exposed to thrombin were used as additional controls in the PMP bioactivity assays.

Susceptibility of *S. aureus* isolates to bactericidal action of PMP. *S. aureus* isolates studied (total, *n* = 28) included organisms obtained from blood cultures of patients with documented staphylococcal bacteremia (*n* = 11) or staphylococcal endocarditis (*n* = 9), as well as common laboratory strains (*n* = 8). The clinical classifications of endocarditis versus nonendocarditis bacteremias were defined as previously described (16). For use in PMP susceptibility assays, *S. aureus* isolates were grown in brain heart infusion broth (14 h, 37°C), harvested by centrifugation, washed twice in normal saline, and resuspended in PBS. PMP was added to *S. aureus* to achieve a final PMP concentration of ~100 U/ml (specific activity, ~12.5 U/mg of protein) and a final bacterial inoculum of 10^3, 10^5, or 10^8 CFU/ml in parallel studies in low-protein-binding microtiter wells (final volume = 200 μl). These *S. aureus* inocula were used to simulate intravegetation bacterial densities observed in early, developing, and well-established endocarditis, respectively (9, 10). At 0, 30, 60, and 120 min of incubation at 37°C, 20-μl aliquots were sampled from each microtiter well, diluted into PBS containing 0.01% SPS (as above), and quantitatively cultured on 6.6% sheep blood agar; curves were then constructed comparing *S. aureus* survival over time. All assays were independently performed in triplicate, and results were recorded as percent survival ± standard deviation. On the basis of our pilot data, we defined a relative breakpoint for *S. aureus* PMP susceptibility as ≤40% survival of the initial inoculum (10^3 CFU/ml) after 2 h of exposure to the PMP preparation. In parallel, the optical densities of *S. aureus*-PMP assay suspensions were monitored spectrophotometrically (as above for *B. subtilis*) to assess PMP-mediated *S. aureus* lysis.

Partial PMP purification by gel filtration chromatography. PMP-rich preparations were partially purified by Sephadex G-50 (exclusion size = 30 kDa; Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) gel filtration chromatography. All glassware involved in chromatographic analysis of PMP preparations was siliconized (Sigmacote; Sigma Chemical Co.) to prevent PMP-glass interaction. The bed (void) volume of the column used (Flex-Column; 2.5 by 50 cm; Kontes Chemistry and Life Science Products, Vineland, N.J.), was calibrated with Dextran B-2000 (2 × 10^6 molecular weight [MW]; Sigma Chemical Co.) and determined to be approximately 85 to 95 ml. Tris-NaCl (TN; 0.05 M Tris-HCl, 1.0 M NaCl; pH 7.5) was used as the column buffer. Two hundred fifty separate 2-ml fractions were collected following the application of 25 ml of PMP preparation to the column. The initial protein concentration of the PMP preparation was ~8.2 mg/ml; therefore, ~205 mg of total protein was initially applied to the column. All fractions collected were tested for protein content spectrophotometrically (as above) and assayed for PMP bioactivity versus *B. subtilis*; selected fractions were also assayed for bioactivity versus representative *S. aureus* isolates, known to be either susceptible or resistant to the killing action of PMP preparations.

SDS-PAGE analysis of PMP preparations. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques were used to analyze both PMP preparations and column-fractionated PMP samples possessing anti-*B. subtilis* bioactivity. Samples were solubilized in SDS sample buffer under reducing (β-mercaptoethanol) conditions in a boiling water bath for 10 min. Approximately 150-μg protein samples were then applied to discontinuous (3.75% stacking, 15% resolving) polyacrylamide gels prepared in 0.375 M Tris-glycine buffer containing 0.1% SDS, as described by Laemmli (17). Following electrophoresis (75 V, 8 h) of PMP preparation samples, resolved protein bands were visualized by Coomassie blue (0.1% R-250 dye in acetic acid-methanol-distilled water [2:5:5]) or formalin-silver (15) staining, and their MWs were estimated by analysis of relative electrophoretic mobility. Low-molecular-mass standards (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) included the α and β chains of insulin (2.3 and 3.4 kDa, respectively,

which comigrate with an apparent molecular mass of 3.0 kDa), bovine trypsin inhibitor (6.2 kDa), lysozyme (LYS) (14.3 kDa), β-lactoglobin (18.4 kDa), carbonic anhydrase (29 kDa), and ovalbumin (43 kDa). Control samples for SDS-PAGE included nonbioactive PMP fractions from column separation, thrombin alone, bacterial growth medium alone, MEM alone, and supernatants of washed PLTs not stimulated by thrombin.

Partial PMP purification by anionic membrane filtration. Leukocyte-derived bactericidal defensins are cationic and purifiable by anionic affinity techniques (6, 12, 22). We thus attempted to partially purify PMPs from thrombin-induced PLT preparations, exploiting their suspected cationic nature, via anionic membrane filtration. Cellulose acetate (CA; 0.22 μm; Costar, Cambridge, Mass.), CA/nitrate (CA/N; 0.45 μm; Millipore Corp., Bedford, Mass.), polysulfonate (0.45 μm; Gelman Sciences, Ann Arbor, Mich.), and asbestos (Seitz Clarifying Hercules C5-L3; Republic Filter, Milldale, Conn.) membranes were used in parallel studies. Aliquots (1 to 10 ml) of thrombin-induced PMP preparations or bioactive column-separated PMP fractions were filtered through siliconized syringe-mounted membranes, and the filtrate was collected. The membranes were washed twice with PBS and then eluted with 2 ml of 1.5 M NaCl-TN buffer (pH 7.2). The filtrates and eluates were then assayed for residual anti-*B. subtilis* bioactivity as previously described; 1.5 M NaCl-TN buffer alone identically filtered through membranes was used as a control in the bioactivity assays.

Partial characterization of PMP by anionic resin absorption. Anionic resin adsorption was used to further investigate the charge characteristics of thrombin-induced PMP preparations. Cellulose phosphate (CP), CA, and SPS resins (all obtained from Sigma Chemical Co.) were used in an effort to bind ionically the bioactive PMP moiety. Thrombin-induced PMP preparations and column-separated bioactive PMP fractions were exposed to CP, CA, or SPS resin at a ratio of 2 U of PMP to 1 mg of resin. PMP-resin suspensions were tumbled for 5 min and centrifuged for 10 min at 10,000 × *g*, and supernatants were collected and tested for residual anti-*B. subtilis* bioactivity. The pellets containing PMP-resin admixtures were then washed twice with PBS and subjected to various eluting solutions, including 1.5 M NaCl (pH 7.2), 1.5 M NaCl (pH 5.5) (adjusted with 1.0 M H_2SO_4), 3 M NaCl, 3 M $CaCl_2$, 5 M potassium acetate, and standard TN buffer. In each case, eluent volumes were equivalent to the original PMP volume adsorbed. Elution suspensions were mixed thoroughly and centrifuged as before to pellet particulate resins, and the supernatants were recovered. Elution supernatants were then assayed for residual bioactivity. Control bioactivity assays were performed with each of the various resin-exposed elution buffers alone against *B. subtilis*.

Adsorption of PMP by PMP-susceptible and PMP-resistant organisms. Thrombin-induced PMP preparations and column-separated bioactive PMP fractions were assayed for residual bioactivity following preadsorption by PMP-susceptible (PMP^s) or PMP-resistant (PMP^r) organisms. Test organisms were grown in brain heart infusion broth (14 h, 37°C), harvested by centrifugation, washed twice in normal saline, and resuspended in PBS to a final inoculum of ~10^8 CFU/ml. *B. subtilis* (PMP^s and LYS resistant [LYS^r]), *S. aureus* 11 (PMP^r), *S. aureus* 19 (PMP^s), and *Micrococcus luteus* (ATCC 4698; LYS^s PMP^r) were each mixed with PMP preparations (final concentration = 10 U/ml) for 30 min at 37°C. Following incubation, PMP-bacteria mixtures were centrifuged for 10 min at 10,000 × *g*; the supernatants were collected and tested for postadsorption anti-*B. subtilis* bioactivity; the postadsorption supernatants were also subjected to SDS-PAGE. Nonadsorbed PMP samples served as controls in both the bioactivity assays and SDS-PAGE analyses.

LYS exclusion. To exclude the possibility that LYS, itself a bactericidal protein present within PLTs, was acting as a contaminating bioactive molecule in PMP preparations, we compared the heat stabilities, anti-*B. subtilis* and anti-*M. luteus* bactericidal activities, and SDS-PAGE protein profiles of PMP preparations with those of commercial LYS alone. Egg-white LYS (Sign a Chemical Co.) was used as a control, representative of PLT LYS, since the two are very similar in MW, specificity, and heat stability (6, 23). Egg-white LYS (ranging from 1 to 10 μg/ml [511 to 5,110 U/ml]), dissolved in either 0.066 M potassium phosphate buffer (pH 6.25) or CP resin-inactivated PMP supernatants, as well as thrombin-induced PMP preparations and bioactive column-separated PMP fractions (100 U/ml), was heated to 50 or 80°C, each for 15 or 30 min. CP resin-inactive PMP preparation supernatants were used as a LYS diluent to control for the possibility that LYS heat inactivation would be reduced in the presence of PMP preparation protein concentrations. After cooling, the heat-treated LYS and PMP samples were immediately assayed for residual bioactivity versus *B. subtilis* and *M. luteus* by the microtiter well assay described above; non-heat-treated LYS and bioactive PMP samples served as controls.

Comparative agar diffusion bioassays of LYS and PMP preparations were also performed. Wells (5 mm) were cut into brain heart infusion agar plates (15 by 100 mm), which were then confluently inoculated with 1-ml suspensions of either 10^8 *B. subtilis* or *M. luteus* cells per ml. Fifty microliters of either egg-white LYS (0.1 to 10 μg/ml in either 0.066 M potassium phosphate buffer or CP resin-inactivated PMP supernatants) or PMP (concentration range = 100 to 200 U/ml) was placed into the wells. The plates were then incubated for 24 h at 37°C, and the zones of growth inhibition were measured. The appearance of zones of inhibition in plates inoculated with *M. luteus* indicated the presence of LYS bioactivity. Similarly, the appearance of zones of inhibition in *B. subtilis*-inoculated assay plates indicated PMP bioactivity. Appearance of anti-*M. luteus* bioactivity surrounding wells containing PMP samples would indicate LYS contamination of such samples. SDS-PAGE was also used to compare the known MW of commercial LYS with that of the bioactive moiety within thrombin-induced PMP preparations and column-separated PMP fractions (as above).

Statistical analyses. Correlation of clinical source of *S. aureus* isolates with (endocarditis versus nonendocarditis) with intrinsic PMP susceptibility was performed by logarithmic regression analysis with BMDP software on the VAX computer mainframe. A probability (*P*) value of ≤0.05 was considered significant for these analyses.

RESULTS

PMP preparation and standardization. Exposure of 10^8 washed PLTs to 1 U of thrombin per ml resulted in mean supernatant protein concentrations of 8.2 mg/ml. The bioactivity of such thrombin-induced PMP preparations versus *B. subtilis* ranged from 150 to 250 U/ml (mean specific activity, ~25 U/mg of protein); control samples containing thrombin alone were found to possess no anti-*B. subtilis* bioactivity. There were no significant differences in bioactivities among

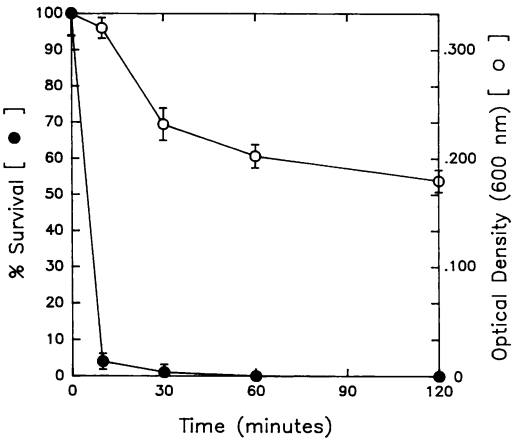

FIG. 1. Comparison of bactericidal and bacteriolytic effects of PMP on *B. subtilis*. Approximately 10^3 *B. subtilis* cells per ml were exposed to 100 U of thrombin-induced PMP per ml (37°C). Percent survival was calculated from quantitative cultures, and optical density was determined spectrophotometrically (λ = 600 nm), with an uninoculated PMP preparation as a spectrophotometer zero. Data represent three independent experiments (error bars represent standard deviation).

PMP preparations obtained from several different rabbit blood donors. Decrease in PMP-exposed *B. subtilis* suspension optical density consistently lagged behind PMP-induced killing (Fig. 1).

Susceptibility of *S. aureus* isolates to the bactericidal action of PMP. At all inocula tested (10^3, 10^5, or 10^8 CFU/ml), the 28 *S. aureus* isolates exhibited heterogeneity in their susceptibilities to PMP (Fig. 2). There was a conspicuous trend between clinical source of the *S. aureus* isolate and PMP resistance. Isolates recovered from patients with endocarditis were more frequently PMP resistant than nonendocarditis isolates; however, this difference did not achieve statistical

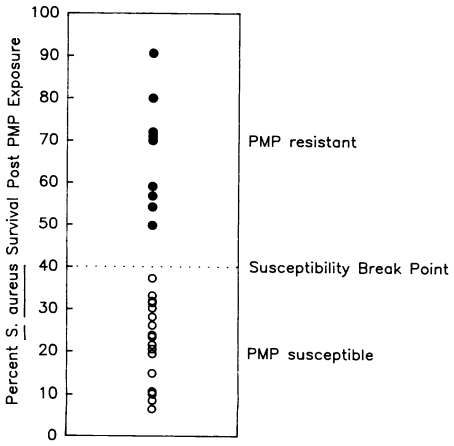

FIG. 2. Distribution of *S. aureus* isolate PMP susceptibility. *S. aureus* isolates were exposed to 100 U of thrombin-induced PMP per ml at inocula of 10^3/ml (37°C); percent survival was calculated following 2 h of PMP exposure. A PMP susceptibility breakpoint was arbitrarily established as ≤40% survival following 2 h of exposure to PMP under the conditions described above. Each datum point represents the mean of at least three independent assays with ≤5% standard deviation (error bars removed for clarification).

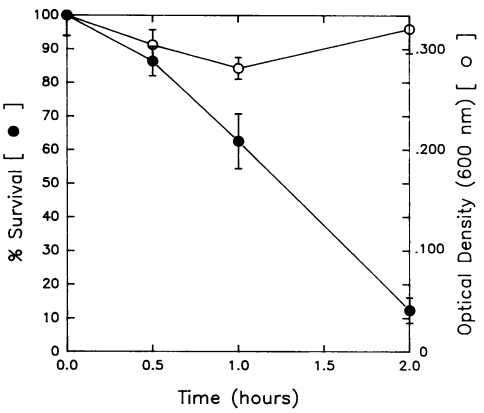

FIG. 3. Comparison of bactericidal and bacteriolytic effects of PMP on a representative PMP-susceptible (PMP^s) *S. aureus* isolate. *S. aureus* ($\sim 10^3$/ml) was exposed to 100 U of thrombin-induced PMP per ml (37°C). Percent survival and optical density were determined as described in the legend to Fig. 2. Data represent three independent experiments (error bars represent standard deviation).

significance (P = 0.27). At an inoculum of 10^3 CFU/ml, staphylococcal survival ranged from a low of 6.5% ± 2.5% for the most PMP-susceptible strain to a high of 90.6% ± 2.1% for the most PMP-resistant strain. Brief sonication and microscopic examination of PMP-exposed *S. aureus* samples confirmed that the reductions in CFU per milliliter were not due to organism aggregation. Based on the PMP susceptibility breakpoint of ≤40% survival of the original inoculum (10^3 CFU/ml) at 2 h, ~67% of *S. aureus* isolates tested were considered PMP^s. Similar heterogeneous susceptibility to the action of PMP was seen at higher *S. aureus* inocula, although the actual percent survival for each respective isolate increased with inoculum size. Each isolate exhibited time-dependent PMP susceptibility, with maximum PMP-induced killing achieved at 2 h (Fig. 3). In contrast to the rapid lysis of *B. subtilis*, PMP-induced lysis of *S. aureus* isolates was prolonged, occurring over the 2-h period monitored. However, similar to results with *B. subtilis*, reduction in viability of *S. aureus* (CFU per milliliter) consistently preceded a decrease in sample optical density, suggesting that PMP-induced *S. aureus* bacteriolysis occurred subsequent to cell death (Fig. 3).

Partial purification of thrombin-induced PMP by column chromatography. Sephadex G-50 column elution fractions 39 to 63 (elution volumes 78 to 126 ml) were found to contain the predominant protein peak (Fig. 4), accounting for ~90% of protein in the original thrombin-induced PMP preparation sample. Of importance, the vast majority of anti-*B. subtilis* and anti-*S. aureus* PMP bioactivities were also localized within fractions 39 to 63, correlating with an estimated molecular mass of ~20 to 35 kDa and indicating that PMP was stable under the conditions of chromatography. Such column elution fractions contained a mean PMP bioactivity of 40 U/ml (mean specific activity of ~32 U/mg of protein). A minor protein peak, possessing anti-*B. subtilis* bioactivity, was also identified (Fig. 4); this protein was estimated to have a molecular mass of ~10 kDa. Control samples containing column elution buffer alone demonstrated no intrinsic bioactivity.

SDS-PAGE analysis of PMP preparations. Thrombin-induced PMP preparations and column-separated bioactive PMP fractions were analyzed for individual protein compo-

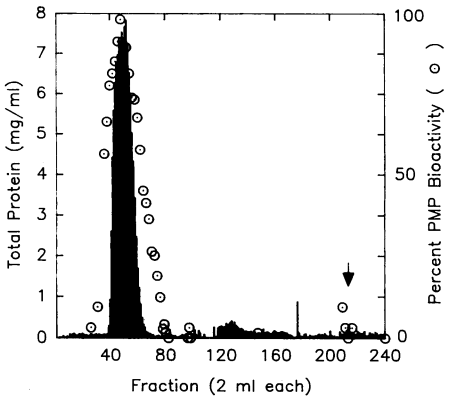

FIG. 4. Sephadex G-50 column elution protein and bioactivity profiles of thrombin-induced PMP. Approximately 205 mg of protein was initially applied to the column (2.5 by 50 cm; bed volume, ~85 to 95 ml; flow rate, ~0.75 ml/min; exclusion size, 30 kDa), which was then eluted with 1.0 M NaCl-TN buffer (see text). Fractions were subjected to assays for total protein and anti-*B. subtilis* PMP bioactivity as described in the text. Shaded curve represents the elution protein profile, while symbols (⊙) indicate the profile of percent PMP bioactivity relative to original, nonfractioned PMP preparations (normalized to 100% PMP bioactivity). Arrow indicates the location of a bioactive minor protein peak, estimated to have a mass of ~10 kDa (see text).

nents by SDS-PAGE. Such bioactive PMP preparations routinely possessed a distinct low-MW (8,500) protein band (Fig. 5), conspicuously absent from nonbioactive gel column elution fractions, as well as from bioinactive anionic membrane or anionic resin filtrates. Such a protein band was also absent from control nonbactericidal samples containing

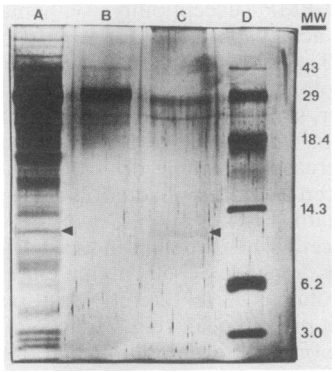

FIG. 5. SDS-PAGE protein profiles of thrombin-induced bioactive PMP (lane A), bioinactive CA/N membrane-adsorbed PMP (lane B), and bioactive 1.5 M NaCl CA/N membrane elution (lane C) samples. Reduced MW standards (10^3) are shown in lane D (see text for specific standards used). Thrombin-induced PMP, CA/N membrane-adsorbed PMP, and CA/N membrane-eluted PMP were prepared and subjected to SDS-PAGE as described in the text. Lanes A, B, and C were each loaded with ~50-μl samples; protein concentrations within these samples were ~150, 25, and 7.5 μg of protein per ml, respectively. Following electrophoresis at 75 V for 8 h, the 15% polyacrylamide gel was stained by the formalin-silver technique (see text). The protein band in the bioactive PMP and PMP CA/N membrane elution samples in lanes A and C only (arrowheads), which migrated with an apparent mass of 8.5 kDa, correlated with PMP bioactivity of these samples, while bioinactive CA/N-adsorbed samples lacked this band (lane B).

TABLE 1. Effect of anionic membrane exposure on bioactivity and SDS-PAGE protein profile of thrombin-induced PMP[a]

Anionic membrane	Relative % PMP bioactivity vs *B. subtilis*				8.5-kDa band in eluate
	Filtration		Membrane elution		
	Preadsorbed	Postadsorbed	PBS	1.5 M NaCl	
CA	100	100	0	0	Absent
PS[b]	100	100	0	0	Absent
CA/N	100	0	0	~100	Present
Asbestos	100	0	0	90	Present

[a] Thrombin-induced PMP preparations were exposed to anionic membranes, tested for residual anti-*B. subtilis* PMP bioactivity, and subjected to SDS-PAGE as described in the text. Preadsorbed PMP bioactivities were normalized to 100%, allowing a relative comparison of preadsorbed versus postadsorbed PMP bioactivity.
[b] PS, polysulfonate.

MEM, thrombin, non-thrombin-exposed PLT supernatants, or bacterial growth medium. This low-MW protein was therefore presumptively identified as thrombin-induced PMP. In no case was a protein band compatible with rabbit LYS (14.3 kDa) detected by SDS-PAGE in bioactive PMP preparations.

Partial purification of PMP by anionic membrane filtration. CA/N and asbestos anionic membrane filters quantitatively removed the anti-*B. subtilis* bioactivity from thrombin-induced PMP preparations, suggesting that the bioactive moiety of PMP is cationic. Near-complete bioactivity was then recoverable by 1.5 M NaCl elution of CA/N or asbestos anionic membranes following exposure to PMP, suggesting that adsorption of PMP occurred via reversible ionic interactions (Table 1). NaCl elution buffer alone, passed over membrane materials, exhibited no bioactivity. Dialysis (cutoff, 3.5 kDa) of anionic membrane eluates against several volumes of PBS to remove the eluting NaCl buffer failed to further enhance the eluate's bioactivity. On SDS-PAGE, the low-MW protein, seen in bioactive PMP preparations, was absent from filtrates recovered from CA/N and asbestos filters (Table 1); SDS-PAGE revealed the presence of this same low-MW protein band in bioactive eluates from CA/N and asbestos membranes (Fig. 5). In no case were such low-MW protein bands found within PBS elution samples of PMP-exposed anionic membranes.

Partial characterization of PMP by anionic resin adsorption. Following adsorption of PMP preparations by CP or SPS (but not CA) resins, all residual bioactivity was removed (Table 2). Of note, despite the complete elimination

TABLE 2. Effect of anionic resin exposure on bioactivity and SDS-PAGE protein profile of thrombin-induced PMP[a]

Anionic resin	Relative % PMP bioactivity vs *B. subtilis*				8.5-kDa band in eluate
	Preadsorption	Postadsorption	Resin elution		
			PBS	1.5 M NaCl	
CA	100	100	0	0	Absent
CP	100	0	0	~15	Present
SPS	100	0	0	0	Absent

[a] Thrombin-induced PMP preparations were exposed to anionic resins, tested for residual anti-*B. subtilis* PMP bioactivity, and subjected to SDS-PAGE as described in the text. Preadsorbed PMP bioactivities were normalized to 100%, allowing a relative comparison of preadsorbed versus postadsorbed PMP bioactivity.

of bioactivity, only ~5% of the total PMP preparation protein content was removed by such anionic resin exposure. Although several elution buffers were tested (including 1.5 M NaCl), no bioactivity could be recovered from PMP-exposed CP and SPS resins, suggesting that PMP(s) was retained by these resins under the elution conditions tested (Table 2).

Adsorption of PMP by PMP-susceptible and PMP-resistant organisms. Highly PMP-susceptible *B. subtilis*, PMP^s and PMP^r *S. aureus* isolates, and PMP^r *M. luteus* were tested for ability to adsorptively remove the bioactive moiety from thrombin-induced PMP preparations. PMP preparations adsorbed by *B. subtilis* exhibited a near-complete loss of residual bioactivity against both *B. subtilis* and *S. aureus* isolates. In contrast, adsorption of PMP preparations by the other organisms failed to substantially remove anti-*B. subtilis* or anti-*S. aureus* bioactivity. Concomitantly, SDS-PAGE analyses revealed loss of the low-MW protein band only from PMP preparations preadsorbed with *B. subtilis* (data not shown).

LYS exclusion. Thrombin-induced PMP and column-separated bioactive PMP preparations were tested for the presence of LYS, to exclude the possibility that LYS was contaminating these preparations and contributing to the observed antibacterial bioactivity. By agar diffusion assay, *B. subtilis* was very sensitive to both thrombin-induced PMP and column-separated PMP preparations (zone size = 15 mm), while no zone of growth inhibition was observed for *M. luteus* around wells containing such PMP preparations. In contrast, zone sizes of 4 to 7 mm were detected around wells containing 0.1 to 10 μg of LYS per ml, respectively, using LYS^s *M. luteus* as the indicator organism; there were no detectable zones of inhibition surrounding wells containing PMP preparations with *M. luteus* as the indicator organism.

Thrombin-induced PMP preparations, as well as bioactive fractions from column chromatography, retained full bioactivity after heating to 80°C for 15 min. In contrast, LYS (10 μg/ml), diluted in buffer alone or in CP resin-inactivated PMP supernatant (to normalize total protein concentration with that of bioactive PMP preparations), significantly lost anti-*M. luteus* activity when exposed for 15 min to temperatures of ≥60°C; such samples heated to 80°C for 15 min exhibited a near-complete loss of anti-*M. luteus* bioactivity. SDS-PAGE comparisons of the bioactive PMP preparations with purified LYS distinguished the characteristic low-MW protein band (8,500) from the larger LYS band (~14,300; Fig. 5) in reduced gels. Moreover, SDS-PAGE demonstrated that anionic membrane filtration, anionic resin adsorption, and *B. subtilis* adsorption each failed to remove the LYS protein band (14 to 15 kDa), while concomitantly removing both anti-*B. subtilis* bioactivity as well as the low-MW protein band from such preparations.

DISCUSSION

The induction of IE is a multistep process in which the interaction of bacteria and PLTs at the cardiac valve surface is felt to be a crucial event. PLTs appear to play both protagonistic and antagonistic roles in initiation of IE, particularly in the presence of endothelium-derived tissue factor (7). From a protagonistic perspective, the adherence of bacteria to PLTs at the surface of damaged valvular endothelium serves as a first step for localized PLT aggregation and eventual vegetation development (9, 20). The subsequent elaboration of tissue factor from either the bacteria-colonized endothelium or subendothelial stroma further propagates the valvular infection by inducing localized procoagulant activity (2, 7, 8). In addition, tissue factor stimulates PLTs to release thrombospondin, a potent enhancer of bacterial adherence to activated PLTs and PLT-fibrin matrices (13).

In support of PLT-mediated bacterial antagonism, recent investigations have shown that tissue factor and thrombin both stimulate rabbit PLT α-granules to release low-MW bactericidal proteins (5) that we have termed PMPs. Human PLTs have also been demonstrated to secrete analogous PMP-like molecules with bioactivities equivalent to that of rabbit PMP (5). Both rabbit and human PMPs exhibit potent antibacterial activity against the viridans streptococci, the most common endocarditis pathogens; moreover, rabbit PMP is suspected of inhibiting the development of streptococcal endocarditis in vivo (5). The present study was designed to determine the basic characteristics of PMP, as well as to define the in vitro bioactivity of PMP against *S. aureus*, the most common intravascular pathogen (19). Rabbit PLTs were used in this study for several reasons: (i) similarities of rabbit and human PLT-derived PMP (5); (ii) availability of rabbits as dedicated PLT donors; and (iii) future use of the well-established rabbit model (1, 10, 11) to further characterize the in vivo role of PMP in IE.

The current investigation demonstrated that there was considerable heterogeneity among *S. aureus* strains in PMP-induced killing, with one-third of the strains found to be PMP resistant. This finding suggests that resistance to PMP is an important virulence determinant in the survival of valve-adherent staphylococci. Also, following exposure to PMP, staphylococcal killing consistently preceded decreases in optical density, suggesting that the mechanism of PMP bactericidal activity primarily involves cell membrane effects leading to cell death, with subsequent cell lysis. Compared with that of *B. subtilis*, both PMP-induced killing and lysis of *S. aureus* were less rapid and complete. The reduction in *B. subtilis* or *S. aureus* CFU per milliliter as a result of PMP exposure was not due to PMP-induced organism aggregation, since ultrasonication strategies prevented bacterial clumping, as confirmed microscopically, without altering cell viability.

Gel filtration chromatography, used to partially purify PMP from thrombin-stimulated PLT preparations, documented the predominant elution protein peak to contain the bioactive PMP moiety. Analytical SDS-PAGE revealed a protein band of 8.5 kDa only in bioactive samples of PMP preparations or PMP-exposed anionic membrane eluates. Of note, a variety of maneuvers, including anionic membrane filtration and preadsorption with *B. subtilis* cells or anionic resins removed both the bioactivity and low-MW protein band from PMP preparations. Based on data from column chromatography, the bioactive PMP fraction (contained within the major protein peak accounting for ~90% of the protein in the original preparation) could be estimated to lie within a range of 20 to 35 kDa. In contrast, SDS-PAGE analyses suggested that the bioactive PMP moiety accounted for only ~5% of the total protein within the original sample and had a molecular mass of only 8.5 kDa. These conflicting data can best be reconciled through the likelihood that PMPs are cationic and are associated with other, possibly anionic, molecules in situ, only resolving from one another by the solubilizing conditions within SDS-PAGE. Of interest, similar cationic, bioactive proteins, such as serum β-lysins, have been demonstrated to be associated with serum carrier proteins (6). Moreover, a minor protein peak, also possess-

ing PMP bioactivity, was chromatographically located in elution fractions 214 to 221 and was found to have a mass of ~10 kDa, nearly identical to that of PMP identified in SDS-PAGE preparations. In addition, such late elution fractions possessing PMP bioactivity were shown by SDS-PAGE to possess the low-MW (8,500) protein band characteristic of PMP. Together, these results indicate that the bioactive protein observed in the minor peak may well represent isolated PMP dissociated from such potential carrier proteins. These data also make it unlikely that PMP is actually a higher-MW protein which is cleaved by proteolysis into smaller fragments (i.e., 8,500 MW) which retain bactericidal activity.

Previously characterized bactericidal proteins, including leukocytic defensins and serum β-lysins, have been shown to be cationic and to possess anionic membrane-directed mechanisms of bactericidal action (3, 12); preliminary studies on PMPs have also supported a cationic charge for these proteins (5). We attempted to exploit this apparent cationic feature of PMPs by utilizing anionic membrane and anionic resin adsorption as partial purification strategies to remove and isolate the bioactive moiety from PMP-rich preparations. CA/N and asbestos membrane filtration substantially removed the bioactive PMP moiety from thrombin-stimulated PLT preparations; 1.5 M NaCl elution led to near-complete recovery of PMP bioactivity from these anionic membranes. In contrast, CP and SPS resins removed most of the PMP bioactivity from PMP-rich preparations but were resistant to elution of the bioactive PMP moiety despite the use of a variety of elution buffers. These observations suggest that bioactive PMP molecules are cationic and have the greatest absorptive affinity for strongly anionic materials with large surface areas (e.g., asbestos or CA/N membranes or CP resin).

PMP preparations adsorbed with *B. subtilis* lost virtually all residual PMP bioactivity against either *B. subtilis* or *S. aureus* isolates, while preparations adsorbed with even the most PMP-susceptible *S. aureus* isolate retained nearly full bioactivity. This difference in PMP activity subsequent to *B. subtilis* versus *S. aureus* adsorption is thus well correlated to the exquisite sensitivity of *B. subtilis* to the bactericidal action of PMP. Compared with the spectrum of *S. aureus* isolates tested, *B. subtilis* was logarithmically more sensitive to PMP-induced lethality, correlating with its ability to adsorptively remove PMP bioactivity.

LYS is a bactericidal protein also contained within PLTs, although stored within organelles distinct from PMP-containing α-granules. Thus, it was important to exclude LYS as a bioactive molecule contaminating the thrombin-induced PMP preparation and contributing to the antibacterial effects observed. To this end, we compared the microbiologic spectra, heat stabilities, and MWs of bioactive PMP with those of well-characterized, commercial LYS, a molecule very similar to rabbit PLT LYS in size, substrate specificity, and heat stability. Bioactive PMP preparations rapidly killed *B. subtilis*, while exhibiting little bioactivity against *M. luteus*; in contrast, LYS was highly bioactive against *M. luteus* and had little activity against *B. subtilis*. Moreover, PMP preparations retained full bactericidal activity against *B. subtilis* despite heating to 80°C for 15 min, while LYS lost most bioactivity against the highly LYS-susceptible *M. luteus* after heating to 60°C for 15 min. Last, comparative SDS-PAGE analyses of bioactive PMP preparations versus exogenous LYS clearly demonstrated the different sizes of these proteins, with LYS consistently exhibiting a size of ~14 to 15 kDa, while PMP exhibited a size of ~8.5 kDa. Collectively, these data provide compelling evidence which virtually excludes LYS as a contaminating, bioactive molecule present in thrombin-induced PMP preparations.

We recently studied the ability of *S. aureus* cells to adhere to and aggregate PLTs in vitro (24). These studies showed that among the same 28 *S. aureus* isolates tested in the present study, there was a highly significant positive correlation between the ability of a staphylococcal strain to adhere to PLTs and that strain's capacity to induce PLT aggregation. In contrast, there was no correlation between staphylococcal susceptibility to the bactericidal activity of PMPs and either PLT adherence or aggregation; this discrepancy suggested that adherence to and aggregation of PLTs by *S. aureus* are covirulence factors, while PMP resistance may be an independent virulence factor of *S. aureus*. Moreover, we observed a clear trend for *S. aureus* isolates which caused IE strains to be more frequently PMP resistant than those strains isolated from non-IE cases. The lack of a statistically significant relationship between PMP resistance on the one hand and the propensity of clinical strains to cause IE on the other hand may indicate that PMP acts in conjunction with other host defense factors to modulate IE. Future evaluation of the mechanism(s) of PMP antibacterial action and the role of PMP in modulating IE await the further purification and characterization of the PMP(s). These investigations are ongoing in our laboratory.

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