Role of Charge and Hydrophobic Interactions in the Action of the Bactericidal/Permeability-increasing Protein of Neutrophils on Gram-negative Bacteria

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ABSTRACT We have recently provided evidence suggesting that the action of purified cationic bactericidal/permeability-increasing protein (BPI) from neutrophils on susceptible gram-negative bacteria requires saturation binding to negatively charged surface sites (Weiss, J., S. Beckerdite-Quagliata, and P. Elsbach, 1980, *J. Clin. Invest.*, **65:** 619–628.)

We now show that this charge interaction is necessary but not sufficient to produce the effects of BPI on the envelope and on viability. By altering the hydrophobic properties of the bacterial (outer) membrane, it is possible to separate saturation binding from the biological action of BPI, indicating that steps beyond surface binding are needed for the antibacterial action. Outer membrane properties were modified by (*a*) reducing temperature during BPI-*Escherichia coli* interaction; (*b*) growing *E. coli* at 42°C to increase the saturated fatty acid content of membrane phospholipids; and/or (*c*) using smooth *E. coli* with a natively less fluid outer membrane. Hydrophobic interaction chromatography on phenyl-Sepharose and measurement of sensitivity to the hydrophobic antibiotic rifampicin were used to monitor the changes in hydrophobic properties of the bacterial outer membrane produced by these manipulations. Nearly all BPI can be removed from the bacterial surface by 80 mM $MgCl_2$ or by trypsin. At 37°C, removal of BPI results in repair of the envelope alterations, but viability is irreversibly lost, even when Mg^{2+} is added after only 15 s of exposure of the bacteria to BPI. However, under conditions of reduced outer membrane hydrophobicity, when saturation binding still occurs within 30 s, *E. coli* can be rescued by addition of Mg^{2+} after up to 5-min exposure to BPI, indicating retardation of postbinding steps.

We conclude that after initial binding BPI must enter into a hydrophobic interaction with the outer membrane in order to produce its antibacterial effects. These postbinding events reversibly mediate the membrane perturbations and irreversibly trigger the bactericidal action of BPI.

INTRODUCTION

We have recently isolated two closely similar membrane-active cationic proteins, one from rabbit and one from human polymorphonuclear leukocytes (PMN), with potent bactericidal activity toward several gram-negative bacterial species (1, 2). The first required step in the antibacterial action of these bactericidal/permeability-increasing proteins (BPI)[1] is saturation binding to the gram-negative bacterial outer membrane (3, 4). Upon binding to susceptible bacteria at 37°C, BPI rapidly produces alterations of the outer membrane, including an increase in permeability to normally impermeant hydrophobic drugs like actinomycin D, and rapidly triggers bacterial killing (1, 2). In contrast, for at least 30 min, there is no detectable disruption of either the structural or functional integrity of the bacterial inner membrane (1, 2).[2]

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[1] *Abbreviations used in this paper:* BPI, bactericidal/permeability-increasing protein; LPS, lipopolysaccharide.

[2] Weiss, J., M. Victor, J. K. Elsbach, and P. Elsbach. Manuscript in preparation.

The remarkably discrete lesions produced by BPI suggest that the locus of BPI action is limited to the bacterial outer membrane. However, while removal of BPI from the surface of *Escherichia coli*, even after 1–2 h, causes reversal of the membrane-perturbing action of BPI, the effect of the protein on bacterial colony formation is irreversible within minutes (5). It appears, therefore, that the action of BPI on the outer membrane and on bacterial viability can be dissociated. In this study we have analyzed further the step(s) in the interaction of BPI with the bacterial envelope and its (their) relation to the membrane-perturbing and bactericidal effects of BPI. The results indicate that after initial binding, obligatory post-binding step(s), whose rate depends on the hydrophobic properties of the bacterial outer membrane, must occur to produce both effects. On the basis of these findings, we propose a scheme for the surface interactions of BPI and possible subsequent events in the bactericidal action of BPI.

METHODS

BPI. BPI was isolated from rabbit PMN as previously described (1).

Bacteria. Three rough strains, W, S15, and J5 (Rc lipopolysaccharide [LPS] chemotype), and one smooth strain, 0111:B4 (parent strain of J5), of *E. coli* were used. They were kindly donated by, respectively, Dr. J. Winter (Department of Microbiology, New York University Medical School), Dr. S. Nojima (University of Tokyo, Japan), and Dr. L. Leive (Laboratory of Biochemical Pharmacology, National Institute of Arthritis, Metabolic, and Digestive Diseases, National Institutes of Health, Bethesda, MD). The three rough strains show virtually identical sensitivity to the bactericidal and permeability-increasing effects of BPI (1). Furthermore, $MgCl_2$ (40 mM) reverses the latter effect in all three strains (5, 6, unpublished observations) indicating that BPI-envelope interactions with all three strains are essentially the same. The bacteria were grown in a triethanolamine-buffered (pH 7.75–7.9) minimal salts medium (7). Stationary phase overnight cultures were transferred to fresh medium (diluted 1:10) and the subcultures were grown to midlogarithmic phase (3–4 h at 37°C; 6–10 × 10^8 bacteria/ml). Bacterial concentrations were determined by measuring absorbance at 550 nm with a Coleman junior spectrophotometer, Coleman Instruments, Inc., Irvine, CA. The bacteria were sedimented by centrifugation at 6,000 *g* for 10 min and resuspended in sterile physiologic saline at the desired concentration.

Anti-BPI antiserum. Generation of goat antibodies against purified rabbit BPI and preparation of IgG-rich fractions have been described (8). Total protein and IgG contents of immune and preimmune IgG fractions are closely similar as judged by agarose gel electrophoresis and immunodiffusion (using rabbit [anti-goat IgG] IgG).

Assay of bacterial viability and outer membrane permeability. Typical (pre)incubation mixtures contained 1–5 × 10^7 bacteria in a total volume of 100–250 μl of sterile physiological saline also containing 8% Hanks' balanced salt solution (HBSS) (without phenol red, Microbiological Associates, Inc., Bethesda, MD), 25 mM Tris-HCl buffer (pH 7.5), 0.1% vitamin-free casamino acids (Difco Laboratories, Detroit, MI) and the indicated amount of BPI.

Bacterial viability. After the indicated preincubation, bacterial viability was measured in one of two ways: (*a*) Aliquots of the bacterial suspensions were serially diluted in sterile isotonic saline and plated on nutrient agar. Bacterial viability was measured as the number of colony-forming units on the plates after incubation at 37°C for 18–24 h; (*b*) Samples of the preincubation mixtures (5 × 10^5 bacteria) were diluted into sterile tubes with 2 ml of nutrient broth containing 0.9% NaCl and 4 mM $MgCl_2$. This concentration of Mg^{2+} prevents further binding of BPI. The samples were incubated 4–5 h at 37°C (~10 generations) until control populations reached late logarithmic phase. Bacterial density was measured by absorbance at 550 nm. Surviving BPI-treated bacteria grow at essentially the same rate as untreated bacteria. Similar results were obtained by the two methods.

Bacterial permeability. The permeability-increasing effect of BPI was measured by determining bacterial susceptibility to actinomycin D (1, 3). *E. coli* are normally resistant to actinomycin D because the outer membrane bars entry of the drug into the cell (9–11). Since bactericidal concentrations of BPI produce little or no inhibition of bacterial protein synthesis for at least 30 min (1), bacterial sensitivity to actinomycin D can be measured by determining bacterial incorporation of [^{14}C]leucine (0.25 μCi/ml; 0.13 mM) (New England Nuclear, Boston, MA) into cold trichloroacetic acid-precipitable material in the presence and absence of 50 μg/ml of actinomycin D (Merck Sharp & Dohme, Division of Merck & Co., Inc., West Point, PA) (3, 12).

Miscellaneous. Protein concentration was determined by the method of Lowry et al. (13).

RESULTS

Removal of surface-bound BPI by trypsin. The antibacterial activities of BPI are directly related to its binding to the bacterial surface (3, 4). When *E. coli* are incubated with saturating amounts of BPI, >99% of the bacteria are rendered nonviable and bacterial protein synthesis is nearly completely inhibited in the presence, but not in the absence, of actinomycin D (1–4, Table I). This actinomycin D-dependent inhibition reflects a permeability-increasing effect of BPI on the bacterial outer membrane that renders *E. coli* sensitive to this normally impermeant hydrophobic drug (3, 10, 11). The outer membrane alteration, but not the loss of viability, can be reversed by incubating BPI-treated *E. coli* with trypsin (Table I) or with ≥40 mM Mg^{2+} or Ca^{2+} (5). These agents initiate repair of the barrier properties by removing surface-bound BPI (4, Table I). Trypsin is a useful agent to study the interaction of BPI with the bacterial envelope. First, trypsin does not affect bacterial viability and permeability to actinomycin D (Table I) and digests only protein exposed on the surface of *E. coli* (14). Second, pretreatment of naked *E. coli* (no BPI) with trypsin does not alter bacterial sensitivity to BPI, indicating that trypsin has no effect on binding sites for BPI. That trypsin-induced repair of the permeability barrier is

TABLE I
Trypsin-induced Reversal of Permeability-increasing Effect of BPI is Accompanied by Removal of Surface-bound BPI

Preincubation	Trypsin added	BPI added	[^{14}C]Leucine incorporation		Viability
			−Act D	+Act D	
		μg			
E. coli alone	−	−	100	110	100
E. coli + BPI (5 μg)	−	−	90	14	<1
E. coli + BPI	+	−	100	80	<1
E. coli alone	−	5.0	95	20	<1
		−	100	110	100
E. coli alone	+	5.0	90	7	<1
E. coli + BPI	+	5.0	80	8	ND*

E. coli W (5×10^7) were preincubated in the standard incubation mixture either alone or with rabbit BPI (5 μg) for 5 min at 37°C. Trypsin (100 μg/ml) was then added, where indicated, and after 15 min at 37°C was inactivated by addition of soybean trypsin inhibitor (200 μg). BPI was then added, as indicated, and bacterial permeability to actinomycin D (Act D) and viability were measured (Methods). Incorporation of [^{14}C]leucine and viability are expressed as percentage of the values obtained with *E. coli* incubated alone. The values shown represent the mean of two closely similar experiments.

* ND, not determined.

associated with removal of the bulk of bound BPI is indicated by the ability of fresh BPI, added after inactivation of trypsin with soybean trypsin inhibitor, to produce once again the permeability-increasing effect. The amount of BPI required to produce this effect is almost identical to the dose needed for *E. coli* not previously exposed to BPI (Table I). This suggests that approximately the same number of binding sites are exposed and, therefore, that trypsin produces essentially complete removal of BPI previously bound to the bacterial surface.

Involvement of irreversible postbinding step(s) in the bactericidal action of BPI. Since the removal of surface-bound BPI by trypsin or by Mg^{2+} (Ca^{2+}) does not result in rescue of the bacteria from the bactericidal action of BPI, even after exposure for only 5 min at 37°C (5, Table I), step(s) beyond surface binding, which are unaffected by trypsin or divalent cations, appear to be involved in bacterial killing by BPI.

To explore any "postbinding" events that may be required for the bactericidal action of BPI, we have used the experimental protocol outlined in Fig. 1. Dilution (>10^3-fold) of *E. coli*-BPI suspensions in isotonic saline containing 4 mM $MgCl_2$ acutely stops further BPI binding without displacing already bound BPI. Since saturation binding is the primary requisite for BPI action (3, 4), binding of BPI must occur during the preincubation for bacterial killing to be apparent after dilution. Mg^{2+}, at high concentrations (≥40 mM), not only prevents further binding but also rapidly (within 1 min [unpublished observations]) removes surface-bound BPI. If irreversible postbinding steps are required for bacterial killing, these steps must also be triggered during the preincubation for bacterial killing to be evident after Mg^{2+} treatment. Under conditions where only binding has occurred during the preincubation, bacterial killing will be apparent after dilution but not after Mg^{2+} treatment, i.e., displacement of bound BPI will "rescue" *E. coli*.

Fig. 2 shows that when rough *E. coli* S15 (grown at 37°C) are preincubated with BPI at 37°C for just 15 s bacterial viability is reduced to <1% of control values and no bacteria can be rescued by Mg^{2+}. Hence, under these conditions, both saturation binding and any subsequent steps must have been initiated within 15 s. To permit detection of putative postbinding steps therefore requires different conditions, in which initiation of the irreversible postbinding phase is retarded without appreciably affecting initial binding, thereby enabling Mg^{2+} to rescue *E. coli* from the bactericidal action of BPI. These conditions are met by carrying out *E. coli*-BPI preincubations at 4°C. At 4°C, apparent BPI-*E. coli* affinity is reduced. However, by increasing the BPI concentration fourfold, sufficient binding occurs within 1 min to produce ~90% killing when measured after dilution (Fig. 2, upper panel). Treatment with Mg^{2+} at this time produces nearly complete rescue (Fig. 2, lower panel). Addition of Mg^{2+} after longer preincubations results in progressively less complete rescue. Transfer of the *E. coli*-BPI

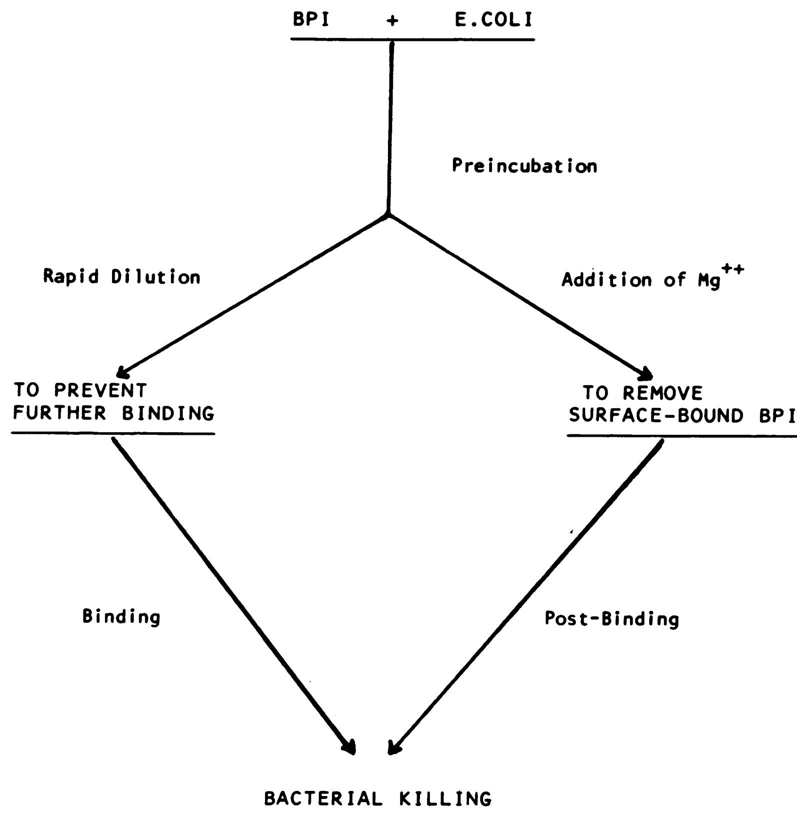

FIGURE 1 Differentiation of binding and postbinding phases of bactericidal action of BPI.

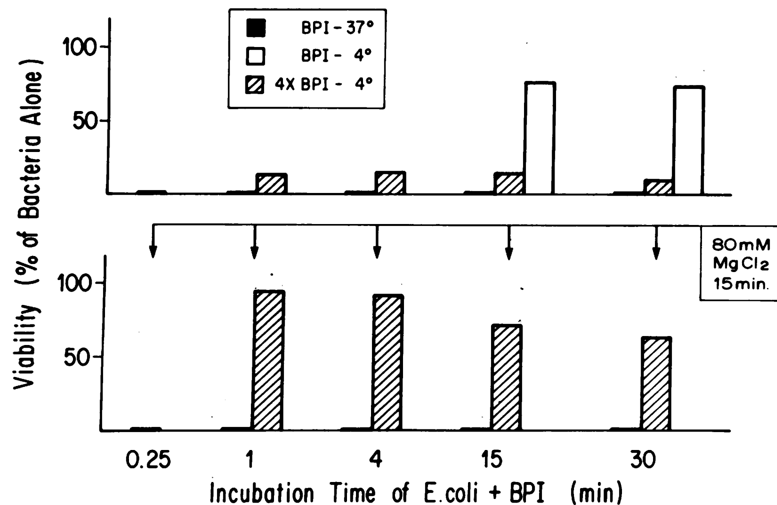

FIGURE 2 Time and temperature dependence of bactericidal action of BPI on *E. coli. E. coli* S15 (5×10^7) were preincubated with 4 μg (solid and open bars) or 16 μg (hatched bars) of rabbit BPI for the indicated period of time either at 37° or at 4°C. At that time, an aliquot was taken to determine bacterial viability (upper panel) and to the remainder of the suspension $MgCl_2$ was added (80 mM final concentration). After further incubation for 15 min, bacterial viability was again determined (lower panel). Results are expressed as percent viability of bacteria incubated alone and represent the mean of two or more similar experiments. In the absence of BPI, bacterial viability is unaffected by 80 mM $MgCl_2$.

suspensions from 4° → 37°C for just 15 s, before adding Mg^{2+}, blocks rescue (data not shown). These findings indicate that binding of BPI to *E. coli* can occur rapidly at both 37° and at 4°C. However, additional step(s) required for bacterial killing that are rapidly triggered by surface-bound BPI at 37° occur at a much slower rate at 4°C.

Role of hydrophobic properties of the outer membrane in the postbinding phase of BPI action. The apparent temperature dependence of the postbinding phase of BPI action could reflect the effect of temperature on the properties of the hydrophobic region of the outer membrane. To examine this possibility we used three different experimental manipulations expected to modify the lipophilic properties of the outer membrane: (*a*) growth of *E. coli* at 42° rather than at 37°C to promote synthesis of membrane lipids with a higher proportion of saturated and longer-chain fatty acids (15, 16); (*b*) preincubation of *E. coli* with BPI at room temperature, i.e., under conditions of reduced bacterial membrane fluidity during initial protein-bacterium interaction; and (*c*) use of smooth *E. coli* (0111:B4) whose long LPS polysaccharide chains are associated with reduced exposure on the surface of hydrophobic groups (8) and reduced outer membrane fluidity (17), compared with rough *E. coli* (J5).

The effect of these experimental conditions on the hydrophobic properties of the outer membrane was monitored by measuring bacterial affinity for the hydrophobic matrix phenyl-Sepharose (Table II) and bacterial sensitivity to the hydrophobic antibiotic rifampicin (Table III).

TABLE II

Effect of Growth, Temperature, and LPS Chain Length on Surface Hydrophobicity of E. coli: Affinity for Phenyl-Sepharose

E. coli strain (LPS chemotype)	Growth temperature	Recovery in gel buffer wash
	°C	%
J5 (Rc)	37	12.8
	42	11.4
0111:B4 (S)	37	109±9.3*
	42	62.0±5.4*‡

E. coli J5 or 0111:B4 (2 × 10^7), prelabeled with [2-^{14}C]uracil during growth at 37° or at 42°C, were applied to phenyl-Sepharose and elution was carried out as previously described (7). The recovery of [2-^{14}C]uracil-labeled bacteria in the gel buffer wash (3–4 vol of isotonic saline containing 5% HBSS and 20 mM Tris-HCl, pH 7.5) is expressed as the percentage of total bacterial radioactivity applied to the columns. The values shown represent the mean (±SEM, where indicated) of two or more experiments.

* The difference in elution of *E. coli* 0111:B4, grown at 37° and 42°C, is statistically significant ($P < 0.01$).

‡ Reapplication of eluted ^{14}C-labeled bacteria to phenyl-Sepharose column again results in retention of ~40–50% of applied bacteria.

Phenyl-Sepharose chromatography of different radiolabeled bacterial populations. Adsorption of radiolabeled bacteria onto phenyl-Sepharose beads has been used to detect differences among bacterial populations in their surface properties (8). The greater the exposure of hydrophobic groups, the greater the affinity of the bacteria for the hydrophobic beads. Thus, rough *E. coli* J5 with short chain LPS are nearly totally retained by the beads (Table II). In contrast, *E. coli* 0111:B4 with shielded hydrophobic groups in the outer membrane are largely recovered in the elution buffer wash. Phenyl-Sepharose chromatography reveals that

TABLE III

Effect of Growth and Incubation Temperature and LPS Chain Length on Sensitivity of E. coli to the Hydrophobic Drug Rifampicin

E. coli strain (LPS chemotype)	Growth temperature	Incubation temperature 37°C (Sensitivity to rifampicin, I_{50})	RT*
	°C	μg/ml[b]	
J5 (Rc)	37	15.3	25.5
	42	18.7	44.4
0111:B4 (S)	37	13.0	43.7
	42	14.4	88.9

E. coli J5 or 0111:B4 (5 × 10^7), grown at 37° or at 42°C, were incubated at 37°C or at room temperature (18–22°C), either alone or with increasing concentrations of rifampicin (7, 14, 29, 58, 116 μg/ml) in the standard incubation mixture that also contained 0.1 μCi [^{14}C]amino acids mixture (>25 μCi/μmol; New England Nuclear). Three to five separate experiments were carried out, providing 15–25 data points for each bacterial population, grown and incubated at the indicated temperatures. These data were analyzed by linear regression, from which the I_{50} of rifampicin (i.e., the concentration of drug required to produce 50% inhibition of bacterial incorporation of [^{14}C]amino acids into trichloroacetic acid-precipitable material [11] and the statistical significance of differences in sensitivity (i.e., of differences between regression coefficients) could be calculated. The following differences in resistance to rifampicin are statistically significant:

(*a*) 0111:B4 (42°/RT)* > 0111:B4 (37°C/RT) ($P < 0.001$).

(*b*) J5 (42°C/RT) > J5 (37°C/RT) ($P < 0.001$).

(*c*) 0111:B4 (42°C/RT) > J5 (42°C/RT) ($P < 0.01$).

(*d*) 0111:B4 (37°C/RT) > J5 (37°C/RT) ($P < 0.001$).

(*e*) All four bacterial populations incubated at room temperature (RT) ≤ same populations incubated at 37°C ($P < 0.05$ for J5 grown at 37°C; $P < 0.001$ for the other populations).

None of the differences in sensitivity between populations incubated at 37°C is statistically significant ($P < 0.10$).

* (Growth temperature/incubation temperature).

the surface hydrophobicity of *E. coli* J5 is unaffected by growth at 42° (vs. 37°C). The surface hydrophobicity of *E. coli* 0111:B4, however, is increased significantly ($P < 0.01$) by growth at 42°C (Table II) as judged by the greater retention of the 42°-grown vs. the 37°C-grown *E. coli* 0111:B4.

Measurement of rifampicin sensitivity. Because the potency of hydrophobic antibiotics such as rifampicin toward *E. coli* is primarily determined by the drug's ability to permeate lipophilic regions of the outer membrane (11), bacterial sensitivity to rifampicin provides an index of the hydrophobic permeability properties of the outer membrane. Table III shows that *E. coli* are much less sensitive to rifampicin at room temperature (18–22°C) than at 37°C. Both growth of *E. coli* at a higher temperature (42° vs. 37°C) and the presence of long-chain LPS (*E. coli* 0111:B4 vs. J5) significantly ($P < 0.001$) reduce bacterial sensitivity to rifampicin at room temperature. At 37°C all four bacterial populations are equally sensitive, presumably because at 37°C their outer membranes are sufficiently fluid, despite the temperature-induced outer membrane changes and the presence of long-chain LPS, to permit penetration of the drug.

The effect of these same experimental conditions on the binding and postbinding phases of BPI action are shown in Table IV. Under all conditions saturation binding of BPI occurs within 30 s. At 37°C postbinding is also triggered within 30 s, except in *E. coli* 0111:B4 grown at 37°C, which show a small delay between binding and postbinding. In contrast, the postbinding phase is significantly delayed at room temperature in three of the four bacterial populations, but not in *E. coli* J5 that were grown at 37°C. The temporal sepa-

TABLE IV

Effect of Growth and Incubation Temperature and LPS Chain Length on Postbinding Phase of BPI Action

			Binding	Postbinding			
			Dilution at:	Addition of Mg^{2+} at:			
E. coli strain	Growth temperature	BPI dose	30 s	30 s	60 s	150 s	300 s
	°C	μg	% viability*	% rescue‡			
Preincubation At 37°C							
J5	37	3	1.0	1.8	<0.1	<0.1	<0.1
	42	3	3.3	<0.1	<0.1	<0.1	<0.1
0111:B4	37	6–8	1.0	23	20	8.2	6.2
	42	6–8	0.4	<0.1	<0.1	<0.1	<0.1
Preincubation At RT							
J5	37	3	0.7	1.3	4.3	0.7	<0.1
	42	6	3.3	71	59	35	12
0111:B4	37	12	1.9	57	41	22	8.1
	42	12	3.9	91	82	60	27

E. coli J5 or 0111:B4 (3×10^7 bacteria), grown at 37° or 42°C, were preincubated at room temperature (RT, 18°–22°C) or at 37°C in 100 μl of standard incubation mixture containing the indicated amount of BPI. At the time indicated, samples were taken and either diluted for measurement of bacterial viability (Methods) or incubated with 80 mM $MgCl_2$ for 15 min at 37°C before dilution and measurement of bacterial viability. The values shown represent the mean of four or more experiments.

* The viability of BPI-treated *E. coli* determined after dilution of the preincubation mixture is expressed as the percentage of viability of *E. coli* preincubated alone.

‡ Rescue of BPI-pretreated *E. coli* by Mg^{2+} is calculated according to the following equation:

$$\% \text{ rescue} = 100 \times \frac{(\% \text{ killing after dilution}) - (\% \text{ killing after } Mg^{2+} \text{ treatment})}{(\% \text{ killing after dilution})}.$$

Statistical analysis (Student's *t* test) shows that the following differences in rescue are statistically significant:

(*a*) 0111:B4 (42°C/RT§) > 0111:B4 (37°C/RT) ($P < 0.05$ at 30 s; $P < 0.02$ at 60, 150, and 300 s).

(*b*) 0111:B4 (37°C/RT) > 0111:B4 (37°C/37°C) ($P < 0.05$ at 30 s).

(*c*) 0111:B4 (42°C/RT) > J5 (42°C/RT) ($P < 0.01$ at 30, 60, and 150 s; $P < 0.05$ at 300 s).

(*d*) 0111:B4 (37°C/RT > J5 (37°C/RT) ($P < 0.01$ at 30 and 60 s; $P < 0.05$ at 150 and 300 s).

(*e*) 0111:B4 (37°C/37°C) > J5 (37°C/37°C) ($P < 0.02$ at 30 and 60 s; $P < 0.05$ at 150 and 300 s).

§ (Growth temperature/preincubation temperature).

ration of the binding and postbinding phases is greatest in 0111:B4 grown at 42°C. More than 50% of the bacteria can still be rescued when Mg^{2+} is added after exposure to BPI for 2.5 min.

Thus, the kinetics of the postbinding phase correlate with the hydrophobic permeability properties of the outer membrane as measured by rifampicin sensitivity (Table III). An apparent exception should be noted, i.e., the slower postbinding phase in the action of BPI at 37°C on *E. coli* 0111:B4 grown at 37°C. This discrepancy may be resolved by the results shown in Table II, indicating that this bacterial population exhibits a lower surface hydrophobicity on phenyl-Sepharose after growth at 37° than at 42°C. Hence these findings actually support the contention that outer membrane hydrophobicity is important in the postbinding phase of BPI action.

Rescue of BPI-saturated E. coli by anti-BPI antibody. Table V shows that an immune (anti-BPI) IgG-rich fraction can also rescue *E. coli* from the bactericidal action of BPI. The rescue elicited by the immune fraction is specifically attributable to anti-BPI antibody since analogous preimmune fractions produce no rescue. There is progressively less rescue when antibody is added after longer *E. coli*-BPI preincubations.

Permeability-increasing effect of BPI also requires postbinding step(s). To determine whether the permeability-increasing effect of BPI also requires postbinding step(s), we measured rescue of *E. coli* J5 and 0111:B4 by Mg^{2+} in the absence and presence of actinomycin D. If the bacteria become permeable to actinomycin D upon surface binding, i.e., before the postbinding steps, the drug will enter and kill the bacteria before the permeability barrier is restored [repair takes 10–15 min (5)] and rescue by Mg^{2+} will be reduced. However, as shown in Table VI, actinomycin D has little or no effect on rescue of *E. coli* J5 or 0111:B4. Thus, initial surface binding of BPI (which occurs within 30 s) does not render *E. coli* sensitive to actinomycin D. Additional postbinding steps must be required for BPI to produce its membrane-active effects.

TABLE V
Ability of Anti-BPI Antibody to Rescue BPI-treated E. coli

Time	Dilution	Additions	
		Anti-BPI IgG	Preimmune IgG
s	*% viability**	*% rescue‡*	
30	9.2	56	<0.1
150	<0.1	21	<0.1
300	<0.1	1.2	<0.1

E. coli J5 (3×10^7), grown at 42°C, were preincubated at room temperature with 6 μg of BPI in 100 μl of the standard incubation mixture. At the times indicated, samples of the preincubation mixture were taken and either diluted for measurement of bacterial viability (Methods) or incubated with immune (anti-BPI) or preimmune IgG fractions (~1 mg IgG fraction/10^7 bacteria) for 20 min at 37°C before dilution and measurement of bacterial viability. The values shown represent the mean of two closely similar experiments.

* The viability of BPI-treated *E. coli* determined after dilution of the preincubation mixture is expressed as the percentage of viability of *E. coli* preincubated alone.

‡ Rescue of BPI-treated *E. coli* by IgG fractions is calculated according to the equation described in Table's IV legend.

DISCUSSION

Fig. 3 provides a tentative scheme of the steps involved in the antibacterial action of BPI toward *E. coli* and other susceptible gram-negative bacteria. The first step is saturation binding of the leukocyte protein to the bacterial outer membrane. The avidity of BPI-outer

TABLE VI
Evidence That Postbinding Steps Are also Required for Permeability-increasing Effect of BPI

Bacteria	Preincubation time	Dilution	Addition of Mg^{2+}	
			−Act D	+Act D
	s	*% viability**	*% rescue‡*	
E. coli J5	30	3.3	77	60
	60	2.2	60	51
	150	ND§	39	38
	300	ND	14	3.3
E. coli 0111:B4	30	3.9	91	92
	60	0.5	83	78
	150	ND	61	43
	300	ND	27	19

E. coli J5 or 0111:B4 (3×10^7 bacteria), each grown at 42°C, were preincubated at room temperature in 100 μl of standard incubation mixture containing 6 or 12 μg of BPI, respectively. At the time indicated, samples were taken and either diluted for measurement of bacterial viability (Methods) or incubated with 80 mM $MgCl_2$ ± 100 μg/ml of antinomycin D (Act D) for 10 min at 37°C before dilution and measurement of bacterial viability. Note that twofold higher than normal concentrations of Act D were used to overcome intrinsically reduced hydrophobic permeability of 42°C-grown bacteria. The values shown represent the mean of two (0111:B4) or four (J5) similar experiments.

* See legend of Table IV.

‡ See legend of Table IV.

§ ND, not determined.

None of the differences in rescue with and without actinomycin D is statistically significant (i.e., $P > 0.05$).

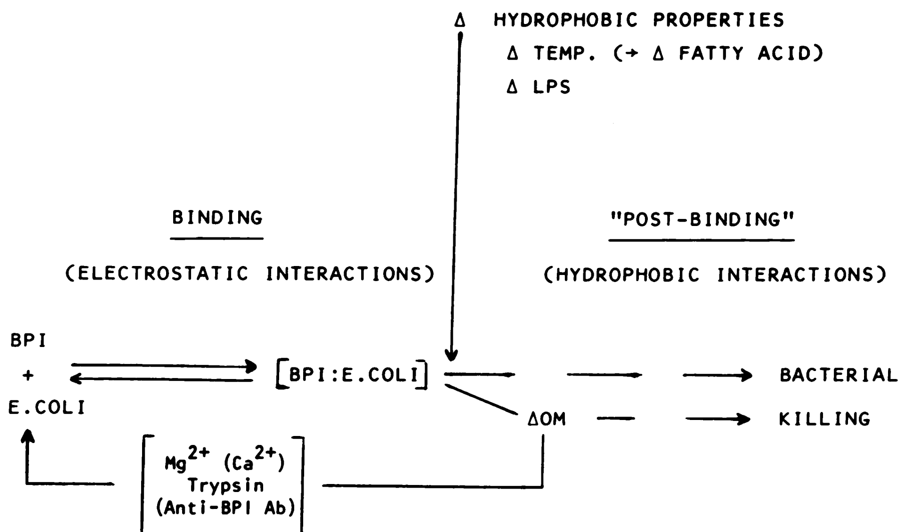

FIGURE 3 Possible steps in the antibacterial action of BPI.

membrane interactions is a primary determinant of the antibacterial potency of BPI toward various strains and species of gram-negative bacteria (3, 4). It is the peculiar affinity of BPI for this unique outer envelope layer of gram-negative bacteria that apparently accounts for the highly specific action of BPI against this group of microorganisms (1, 2). Initial protein attachment seems principally mediated by electrostatic attraction between the cationic protein and anionic bacterial surface sites (4, 8). The predominant negatively charged components of the outer leaflet of the outer membrane are LPS (11, 18). Because of their relative abundance, peripheral localization, and almost exclusive presence in gram-negative bacterial outer membranes, we believe that the anionic groups in the LPS provide the initial attachment sites for BPI (4).

As shown in this study, under certain conditions, removal of BPI from the bacterial surface can prevent its biological action. Thus, saturation binding, while necessary, is not sufficient to generate the antibacterial effects of BPI. According to our model (Fig. 3), saturation binding is required to trigger "postbinding" steps that are actually responsible for the membrane-active and bactericidal effects of BPI.

This second phase of action can be slowed either by reducing the temperature during initial protein-bacterium interaction and/or by modifying the chemical composition of the bacterial (outer) membrane. Because the delay in the postbinding phase is produced under conditions when hydrophobic interactions with the outer membrane are impeded (Tables II–IV), we propose that (rate-limiting?) postbinding steps involve hydrophobic interactions between BPI and the outer membrane. Other properties of the bacteria that may be affected by the conditions used to delay postbinding do not seem to play an important role in this phase of BPI action. Thus, we cannot readily account for the differences in the speed of postbinding steps among 37° and 42°C-grown *E. coli* J5 and 0111:B4, shown in Table IV, by invoking general effects of temperature on metabolic activity (also note that in *E. coli* J5 grown at 37°C the speed of postbinding steps is not detectably slowed by reducing incubation temperature from 37° to ~20°C) or in other bacterial properties that are unrelated to envelope hydrophobicity. Analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the outer membrane proteins of *E. coli* J5 and 0111:B4, grown at 37°C or at 42°C, reveals no recognizable qualitative or quantitative differences (data not shown), further suggesting that the predominant effects of varying growth and incubation temperature on BPI action can be explained by modification of hydrophobic components of the (outer) membrane.

In the leukocyte, BPI is tightly (granule) membrane associated (2, 3) indicating that this protein possesses lipophilic properties. The anionic groups of the LPS are clustered in close proximity to lipid A, the moiety through which the LPS are embedded into the hydrophobic interior of the outer membrane (11). Thus, primary charge-charge interactions between BPI and outer membrane LPS may be quickly followed by secondary hydrophobic interactions allowing rapid transition from the binding to the postbinding phase of BPI action.

In analogy with recent studies on the interaction of terminal components of complement with the envelope of *Salmonella minnesota* (19) hydrophobic interactions may also serve to stabilize initial BPI at-

tachment. This is suggested by the reduction in apparent affinity of *E. coli* for BPI observed when the postbinding phase is rendered more difficult (Table IV). The high concentration and close proximity of anionic and hydrophobic sites provided by the LPS may produce the strong affinity between BPI and the outer membrane. The capacity to engage in hydrophobic, as well as ionic, interactions may also account for the far greater affinity and potency of BPI than of other highly cationic leukocyte proteins toward gram-negative bacteria.

The nature of the envelope effects generated by BPI also suggest that the protein interacts with hydrophobic regions of the envelope. The outer membrane of enteric bacteria like *E. coli* is unusual in its resistance to permeation by small hydrophobic molecules such as actinomycin D and rifampicin (11). The apparent structural basis of this permeability barrier is the absence of phospholipid bilayer regions, purportedly a consequence of a highly asymmetric membrane organization in which phospholipids are excluded from the outer leaflet by densely packed LPS and proteins (11). The postbinding events that mediate BPI permeability-increasing effect may involve a rearrangement of phospholipids and their appearance in the outer leaflet. BPI renders outer membrane phospholipids susceptible to degradation by endogenous and certain exogenous phospholipases A (1, 2, 20), indicating that the state of these envelope phospholipids is, indeed, altered by BPI. The membrane-perturbing effects of BPI require the continued presence of the protein at surface sites because its removal promptly initiates reversal of the outer membrane alterations, including those involving phospholipids (5).

In contrast, the bactericidal action of BPI can apparently proceed without surface-bound protein after a previous interaction as brief as 15 s. Thus, surface-bound BPI triggers both reversible outer membrane alterations and irreversible (i.e., not reversed upon removal of surface-bound protein) postbinding steps that lead to bacterial killing. The nature of these irreversible steps including any possible relation to the reversible outer membrane changes is unknown. One possibility is that surface-bound BPI activates bacterial "autolytic" enzyme(s). In fact, attachment of BPI results in net degradation of phospholipid and peptidoglycan in *E. coli* (1, 2, 20, unpublished observations). However, BPI kills phospholipase-less mutants of *E. coli* (1, 2, 20) and net peptidoglycan degradation is halted upon release of surface-bound BPI (unpublished observations). This means that phospholipid degradation is not essential for killing and that any role for peptidoglycan turnover in the bactericidal action of BPI must be provided by very limited degradation.

An alternative possibility is that bacterial killing is brought about by a small lethal subpopulation of bound BPI that penetrates beyond the bacterial surface to sites from which the protein cannot be removed by Mg^{2+} or trypsin. The initial requirement for saturation binding may then simply reflect the low efficiency (frequency) of protein penetration. The ability of anti-BPI antibody to rescue BPI-saturated *E. coli* might reflect then the complexing of BPI by antibody at the bacterial surface, thereby preventing further protein translocation. However, we have not excluded the possibility that antibody removes rather than "freezes" surface-bound BPI.

The multiphased sequence of BPI action, initiated by reversible interaction with the bacterial surface and followed by irreversible steps leading to cell death is reminiscent of the action of various toxins, including several bactericidal proteins secreted by bacteria within the family of Enterobacteriaceae (21, 22). Many of these bacteriocins are also highly cationic proteins, of molecular size similar to that of BPI, and possessing antibacterial activity specifically directed against certain gram-negative bacteria. A striking feature of the structure/activity relationships of the bacteriocins is the localization of their binding, entry, and bactericidal functions into separate "domains" of the proteins (21). It will be of interest in the future to determine whether the binding, membrane-active, and bactericidal functions of BPI are similarly segregated within separate structural regions of the protein. Such studies may reveal whether BPI is a mammalian analogue of the bacteriocins, representing a highly effective structural design for a bactericidal protein vs. gram-negative bacteria that has been conserved through evolution.

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