JCI The Journal of Clinical Investigation

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J Clin Invest. 1991;88(3):750-754. https://doi.org/10.1172/JCI115372.

Research Article

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Penicillin-binding Protein Inactivation by Human Neutrophil Myeloperoxidase

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Abstract

Myeloperoxidase (MPO), H_2O_2, and chloride comprise a potent antimicrobial system believed to contribute to the antimicrobial functions of neutrophils and monocytes. The mechanisms of microbicidal action are complex and not fully defined. This report describes the MPO-mediated inactivation, in *Escherichia coli, Staphylococcus aureus,* and *Pseudomonas aeruginosa,* of a class of cytoplasmic membrane enzymes (penicillin-binding proteins, PBPs) found in all eubacteria, that covalently bind β-lactam antibiotics to their active sites with loss of enzymatic activity. Inactivation of "essential" PBPs, including PBP1–PBP3 of *E. coli,* leads to unbalanced bacterial growth and cell death. MPO treatment of bacteria was associated with loss of penicillin binding by PBPs, strongly suggesting PBP inactivation. In *E. coli,* PBP inactivation was most rapid with PBP3, where the rate of decline in binding activity approximated but did not equal loss of viability. Changes in *E. coli* morphology (elongation), observed just before bacteriolysis, were consistent with early predominant inactivation of PBP3. We conclude that inactivation of essential PBPs is sufficient to account for an important fraction of MPO-mediated bactericidal action. This feature of MPO action interestingly recapitulates an antibacterial strategy evolved by β-lactam–producing molds that must compete with bacteria for limited ecologic niches. (*J. Clin. Invest.* 1991. 88:750–754.) Key words: phagocytes • microbicidal mechanisms • *Escherichia coli* • β-lactam antibiotics • *Staphylococcus aureus*

Introduction

Myeloperoxidase (MPO)[1], an enzyme found in the azurophil granules of neutrophils, is a major component of the oxygen-dependent antimicrobial systems of phagocytes (1). When combined with H_2O_2, synthesized by the respiratory burst, and chloride, it forms a number of powerful oxidants (2, 3). Lesions induced by MPO-derived oxidants have been best characterized for sites at the bacterial cytoplasmic membrane. These include loss of energy charge and nutrient transport (4). Determining whether any of these effects is either necessary or sufficient to account for loss of microbial viability has been an elusive goal.

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Received for publication 16 August 1990 and in revised form 15 March 1991.

1. *Abbreviations used in this paper:* MPO, myeloperoxidase; PBP, penicillin-binding protein.

J. Clin. Invest.
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0021-9738/91/09/0750/05 $2.00
Volume 88, September 1991, 750–754

Penicillin-binding proteins (PBPs), found in the cytoplasmic membrane of all eubacteria (5), mediate the synthesis, degradation, and remodeling of peptidoglycan cell wall components (6, 7). β-lactam antibiotics act by binding to and inactivating PBPs, disrupting balanced PBP activity and resulting in bacterial death. Clinically important antibiotic resistance may be due to the emergence of PBPs with a lower affinity for β-lactams (8–13).

Because PBP inactivation is microbicidal and because PBPs are located in the bacterial cytoplasmic membrane at sites potentially accessible to MPO-derived oxidants, we investigated the effects of the MPO system on the penicillin-binding properties of *Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus,* and compared PBP inactivation with loss of microbial viability.

Methods

Special reagents. Human MPO was prepared from leukocytes obtained from a patient with chronic myelogenous leukemia, as previously described (14). Other reagents were obtained from Sigma Chemical Co., St. Louis, MO, except xanthine oxidase (Boehringer Mannheim, Indianapolis, IN), benzyl penicillin *N*-ethyl piperidine salt, [phenyl-^{3}H] and EN^{3}HANCE (Du Pont Co., Wilmington, DE).

Bacterial strains. Escherichia coli (ATCC 11775; American Type Culture Collection, Rockville, MD), *Pseudomonas aeruginosa* (ATCC 27853), and *Staphylococcus aureus* (502A) were maintained on blood agar plates, grown overnight in trypticase soy broth, then diluted 50–100-fold and grown for 3–4 h. Organisms were harvested in log phase, washed twice, and suspended to the required absorbance at 540 nm.

Microbicidal systems. MPO: Nonenzymatic components indicated in the legends to figures were prewarmed in 125-ml flasks in an oscillating water bath at 37°C, and bacteria were added ~ 1 min before the initiation of the reaction with MPO and glucose oxidase. At intervals, the reaction was stopped with 1 mM azide, viable bacteria were determined by the pour plate method (15), and cells were pelleted and stored at −80°C.

Xanthine oxidase or gentamicin. E. coli were exposed to 50 μg/ml gentamicin or to a xanthine oxidase/acetaldehyde microbicidal system described in the legend to Fig. 1 *B* (16). At intervals, samples were removed for viability determination and cells were pelleted immediately and stored at −80°C.

Membranes. For *E. coli* or *P. aeruginosa* frozen cells were thawed, washed once, and suspended in 30 mM Tris, pH 8.0, containing 5 mg/ml lysozyme and 10 mM K-EDTA. After 10 min on ice, samples were incubated with 10 μg/ml DNAse, 10 μg/ml RNAse, and 20 mM $MgSO_4$ for an additional 10 min. Membranes were collected by centrifugation at 50,000 *g* for 30 min at 4°C and suspended in 50 mM sodium phosphate, pH 7.0, to a protein concentration of 10–20 μg/ml (17).

S. aureus membranes were prepared by incubating thawed cells suspended in 50 mM potassium phosphate, pH 7.9, with 100 μg/ml lysostaphin, 10 μg/ml DNAse, 10 μg/ml RNAse, and 10 mM $MgSO_4$ at 37°C for 30 min. Membranes were pelleted at 50,000 *g* for 60 min at 4°C and suspended in 50 mM sodium phosphate, pH 7.0, to a protein concentration of 5–10 μg/ml.

Penicillin-binding proteins. Bacterial membranes were incubated with 5 μg/ml benzyl penicillin *N*-ethyl piperidine salt, [phenyl-^{3}H] for 10 min at 37°C. Sample buffer (18) was added and samples were

boiled for 5 min. SDS-PAGE was performed using 8% running gels, 4% stacking gels, at 50 V for 16 h, with 200 μg protein per lane. Gels were stained with Coomassie blue and prepared for fluorography with EN^{3}HANCE. Examination of Coomassie-stained gels before addition of EN^{3}HANCE showed no difference in protein staining between lanes. Dried gels were placed next to preflashed Kodak X-omat AR-2 film at −80°C for 2–3 mo. Densitometry was performed using a video densitometry and analysis system (Visage 2000; BioImage Corp., Ann Arbor, MI), and PBP activity for each band was expressed as the percentage (mean±SE) of the integrated band intensity at 0 min.

Bacterial morphology. E. coli or *P. aeruginosa* were incubated with the microbicidal system of interest and, at intervals, samples were applied to glass slides, air dried, heat fixed, and stained with safranine for 60 s. Slides were examined with a laser scanning confocal microscope (MRC-500; Bio-Rad Microscience, Cambridge, MA) and the lengths of 30 organisms/sample were determined. Results were averaged for three separate experiments and data were compared using Student's two-tailed *t* test.

Results

Incubation of *E. coli* with a microbicidal system consisting of human MPO, H_2O_2, and chloride, resulted in progressive loss of viability, which was not observed when sodium chloride was replaced by isotonic sodium sulfate. At intervals, *E. coli* were analyzed for membrane penicillin binding activity as described in Methods. Each of the expected PBPs, designated 1–7b, was detectable at the beginning of the incubation (Fig. 1 *A,* 0 min), but penicillin binding was completely lost after an hour's incubation with the complete MPO system (Fig. 1 *A,* 60 min). This effect was not attributable to long-lived oxidants (3) since addition of reducing agents, 1 mM 2-mercaptoethanol, dithiothreitol, or thiosulfate, appeared to have no effect on the relationships between PBP activity and microbial viability (data not shown). PBPs from *E. coli* exposed to the control MPO system were not significantly affected (Fig. 1 *A,* control 60). PBPs were not significantly affected by microbicidal systems mediated by gentamicin or the oxidizing system xanthine oxidase/acetaldehyde (Fig. 1 *B*), indicating that PBP inactivation was not a simple consequence of cell death.

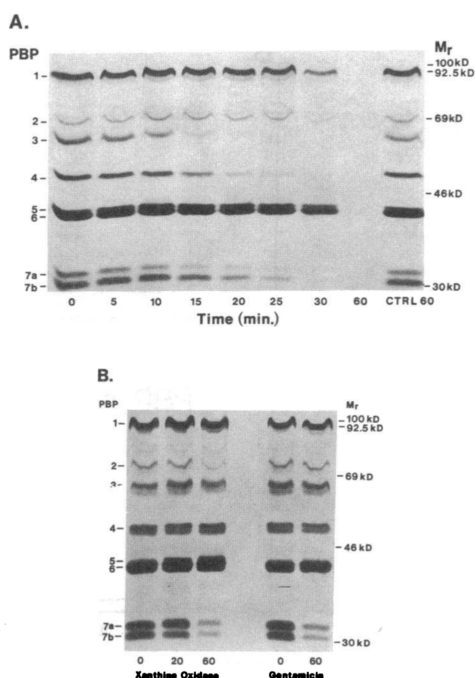

Figure 1. Effect of microbicidal systems on *E. coli* penicillin-binding proteins. (*A*) *E. coli* were exposed to a microbicidal MPO system containing (per ml) 10^9 bacteria, 0.18 U of MPO, 0.23 U of glucose oxidase, 0.1 M NaCl, 0.04 M Na acetate, pH 5.0, 0.01 M glucose, 0.01 M Na_2SO_4, and 0.05 mg gelatin (27) for 0–60 min. For the control system, sodium chloride was replaced with 0.067 M sodium sulfate (control 60). At indicated intervals samples were labeled with ^{3}H-benzyl penicillin and fluorographed as described in Methods. (*B*) *E. coli* were exposed to 50 μg/ml gentamicin or a microbicidal xanthine oxidase/acetaldehyde system containing (per ml) 4×10^7 *E. coli,* 0.075 M Na phosphate, pH 7.0, 0.05 M Na_2SO_4, 15 μM $FeSO_4$, 0.06 U xanthine oxidase, and 0.75 μl acetaldehyde (16). PBPs are numbered to the left according to Spratt (28), mobility of molecular size markers are indicated to the right, and time of incubation is on the bottom.

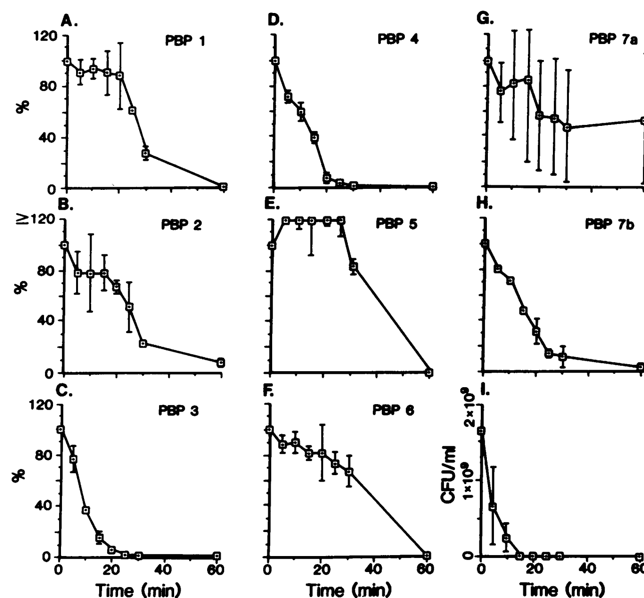

Figure 2. Inhibition of *E. coli* penicillin binding by MPO-derived oxidants. (*A*) PBP1; (*B*) PBP2; (*C*) PBP3; (*D*) PBP4; (*E*) PBP5; (*F*) PBP6; (*G*) PBP7a; (*H*) PBP7b; (*I*) microbial viability. PBP binding activity was expressed as the percentage (mean±SE, $n = 2$) of the integrated band intensity at 0 min.

Rates of MPO-mediated inactivation among PBPs varied considerably (Fig. 2). Binding activity of PBPs 3, 4, and 7b (Fig. 2, *C, D,* and *H*) declined most rapidly, with 50% loss of activity after 10–15 min exposure. PBPs 1, 2, 5, 6, and 7a (Fig. 2, *A, B, E, F,* and *G*) were the most resistant to MPO, with retention of > 50% activity after 25–60 min. The decline in *E. coli* viability induced by MPO is shown for comparison in Fig. 2 *I. E. coli* viability in the xanthine oxidase/acetaldehyde system was: 0 min = 4.0×10^7 CFU/ml, 20 min = 2.3×10^7, 60 min = 1×10^2; for gentamicin: 0 min = 1.68×10^8, 60 min = 1×10^2.

Similar results were obtained with *P. aeruginosa* (Fig. 3) and *S. aureus* (Fig. 4). Each of the PBPs, labeled 1a–5 for *P. aeruginosa* and 1–4 for *S. aureus,* was present at 0 min and had almost completely disappeared by 60 min incubation with the complete MPO system, while exposure to the control MPO system had no effect. A new band of penicillin binding activity in *P. aeruginosa,* labeled PBP4′ (Fig. 3) appeared after 5 min exposure to the MPO system, and declined from 10 min onwards.

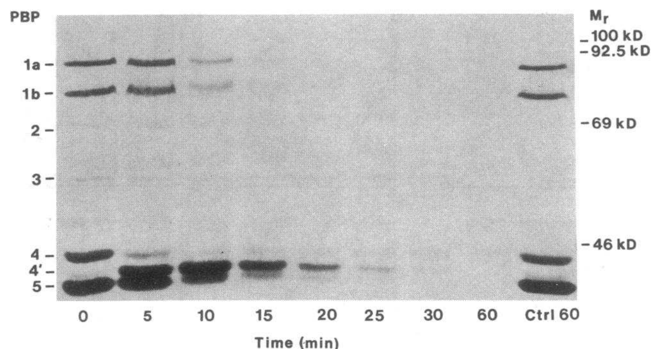

Figure 3. Effect of the MPO system on *P. aeruginosa* PBPs. The MPO system was as described in Fig. 1, except that the bacteria was *P. aeruginosa*, the [MPO] = 0.30 U/ml, and the reaction mixture also contained 1 mM $MgSO_4$.

P. aeruginosa PBPs 1a, 1b, 4, and 5 declined rapidly, (Fig. 5, *A, B, E,* and *G*) with 50% loss of activity after 0–10 min exposure, while PBPs 2 and 3 retained 50% of their activity until 20–30 min (Fig. 5, *C* and *D*). *S. aureus* PBPs 1, 3, and 4 (Fig. 6, *A, C,* and *D*) also fell very rapidly in response to MPO, with only 0–10% activity remaining after 5 min, and PBP2 declined more slowly (Fig. 6 *B*). The loss of bacterial viability in response to the MPO system for *P. aeruginosa* and *S. aureus* can be seen in Fig. 5 *H* and Fig. 6 *E*, respectively.

For *E. coli*, loss of microbial viability correlated best with loss of penicillin binding activity of PBP3 ($r = 0.91$). For *P. aeruginosa*, good correlations were found for PBPs 1a, 1b, and 5 ($r = 0.91$, 0.94, and 0.91, respectively). For *S. aureus*, loss of viability correlated best with a decline in PBP2 ($r = 0.93$), though inactivation of PBPs 1, 3, and 4 actually preceded killing.

In *E. coli*, selective, antibiotic-mediated inactivation of PBP3 results in the formation of long filamentous cells (19), forming a basis for the view that PBP3 is important for septation of the cell wall during division (20). The early, predominant inactivation of PBP3 after MPO exposure suggested that growth observed during or shortly after incubation with the MPO system might result in the formation of unusually long rods. Indeed, microscopic examination indicated an increase in the average length of MPO-treated *E. coli*, from a baseline value of 1.56±.03 (SE) μm, by 0.12±.04 μm at 15 min ($P < 0.05$) and 0.19±.05 μm at 30 min ($P < 0.01$). Illustrative

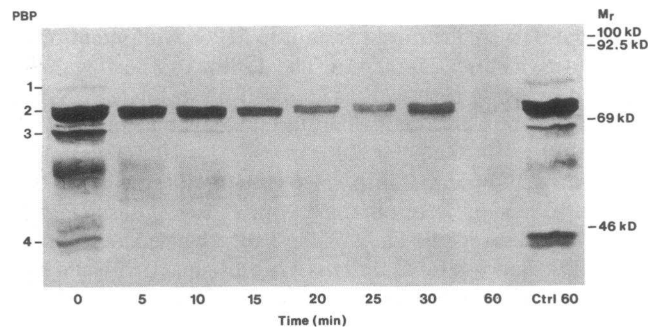

Figure 4. Effect of the MPO system on *S. aureus* PBPs. The MPO system was as described in Fig. 1, except that the bacteria was *S. aureus*, the [MPO] = 0.08 U/ml, the [glucose oxidase] = 0.20 U/ml, and there was no gelatin.

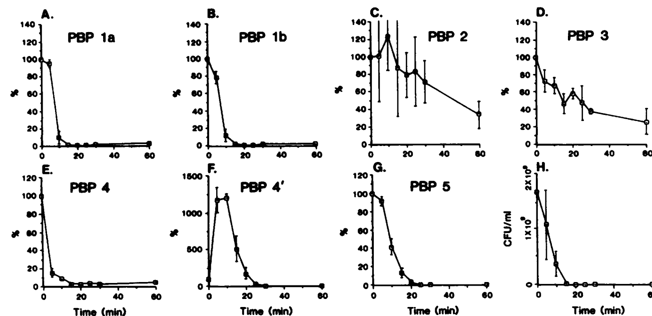

Figure 5. Inhibition of *P. aeruginosa* PBP activity after MPO exposure. (*A*) PBP 1a; (*B*) PBP 1b; (*C*) PBP2; (*D*) PBP3; (*E*) PBP4; (*F*) PBP4'; (*G*) PBP5; (*H*) microbial viability.

photomicrographs are reproduced in Fig. 7 and show one of the long filamentous forms occasionally observed in the MPO exposed group. Although changes in length could have been secondary to other factors such as nutritional (21) or nonspecific oxidant stress (22), neither filamentous forms nor overall increases in cell length were observed among organisms treated for 30 min either with buffered chloride (change = 0.00±.04 μm), with the chloride deficient, control MPO system

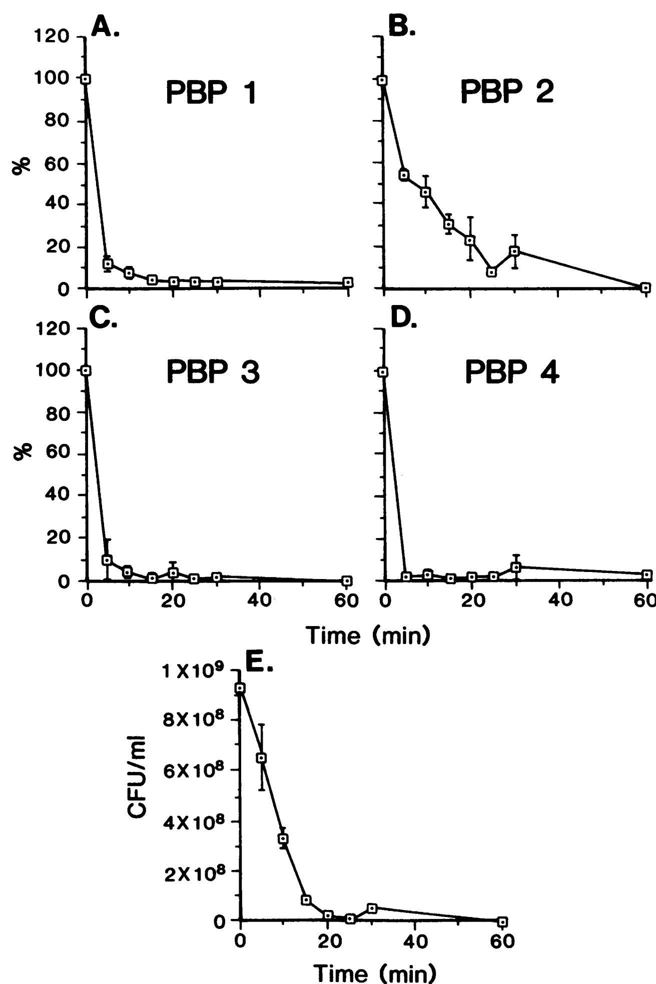

Figure 6. Inhibition of *S. aureus* PBP activity after MPO exposure. (*A*) PBP1; (*B*) PBP2; (*C*) PBP3; (*D*) PBP4; (*E*) microbial viability.

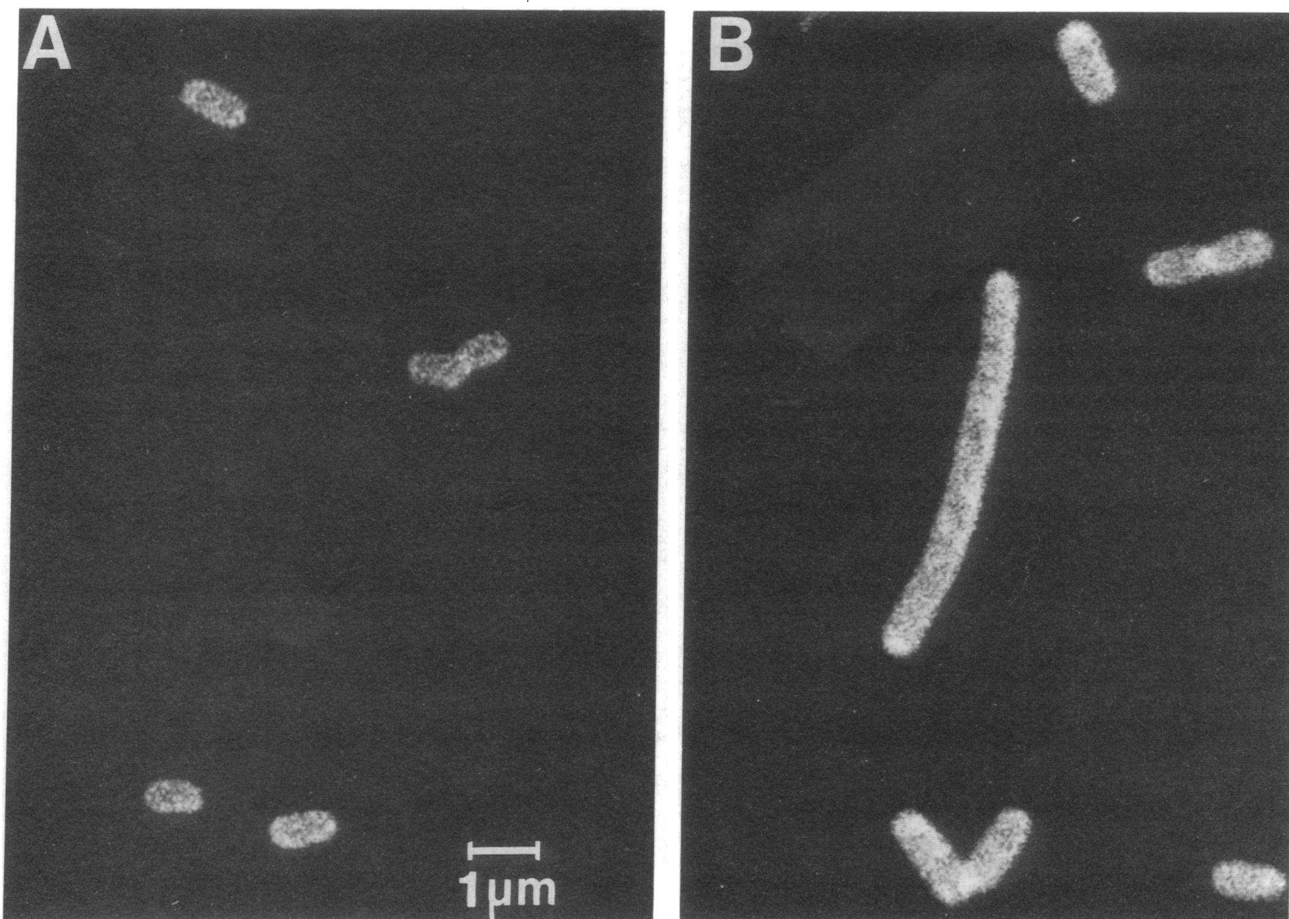

Figure 7. E. coli morphology after exposure to the MPO system. Photomicrographs of *E. coli* after (*A*) 0 min and (*B*) 30 min incubation with the MPO system, as described in Fig. 1. *E. coli* were stained with safranine and examined as described in Methods.

(0.07±.04 μm), with 50 μg/ml gentamicin (0.11±.06 μm) or with the xanthine oxidase/acetaldehyde system (0.01±.06 μm). Thus the morphologic change was specifically related to active MPO exposure. The degree of elongation produced by the MPO system was not as dramatic as can be observed with PBP3-selective antibiotics, but MPO-mediated damage to other PBPs and to microbial metabolism at other sites (4) may have limited further growth and elongation of the organisms. *P. aeruginosa* PBPs are thought to have similar functions to their correspondingly numbered counterparts in *E. coli. P. aeruginosa* showed no change in length after MPO exposure (baseline = 2.03±.04 μm, change at 30 min = 0.01±.04 μm), consistent with the lack of significant early PBP3 inactivation in *P. aeruginosa.*

Discussion

The neutrophil-derived MPO system has antimicrobial effects against a broad range of microorganisms (1). The precise nature of the lesions leading to bacterial death have been difficult to define. Here, we have demonstrated that the MPO system inhibits the penicillin-binding activity of multiple bacterial PBPs. The loss of penicillin binding very likely reflects enzymatic inactivation of the PBPs. In rare instances, however, using specially selected mutants, enzymatic activity and penicillin binding can be dissociated (23). MPO-mediated PBP inactivation does not occur with a control MPO system, lacking chloride, in which H_2O_2 and MPO are present, but no killing takes place. Nor is the effect simply a nonspecific manifestation of cell death, since bactericidal systems containing gentamicin or xanthine oxidase/acetaldehyde are equally ineffective in inactivating PBPs. Thus, PBP inactivation is related to exposure to a bactericidally active MPO system.

Relationships between inactivation of PBPs and loss of viability may be complex (5, 23). In *E. coli,* where it has been best studied, each high molecular weight PBP: 1, 2, and 3, has been considered essential to survival under usual growth conditions, while PBPs 4, 5, and 6 have not. Inactivation of PBP 1 or 2 by antibiotics usually produces cell lysis (5) while PBP3 inactivation may result in loss of viability independent of cytolysis (24). MPO-mediated killing of *E. coli* correlated best with loss of PBP3 activity, although inactivation of no single PBP could account fully for microbicidal activity. An MPO-mediated distortion of the overall pattern of PBP synthetic and remodeling functions, rather than inhibition of a single PBP, may be responsible for cell death. Indeed, synergistic antibiotic-induced microbicidal effects involving inhibition of multiple PBPs have been described previously (23). Analagous synergistic events are likely to play a role in MPO-mediated antimicrobial activity.

E. coli morphology is affected by the MPO system. An increase in *E. coli* length is seen with progressive exposure to

MPO. Once again, this process occurs solely in the presence of an active MPO system, since neither the control MPO system nor microbicidal systems containing gentamicin or xanthine oxidase/acetaldehyde have significant effects on cell length. This progressive lengthening of *E. coli* is consistent with the preferential inhibition of PBP3 induced by MPO, since the latter should result in a diminution in the synthesis of cross walls while cylindrical wall synthesis continues.

The MPO system inactivates PBPs in a variety of bacteria, including Gram-negatives (*E. coli* and *P. aeruginosa*) and a Gram-positive (*S. aureus*). However, the pattern of PBP inactivation differs amongst bacterial species. While PBP3 is inactivated most rapidly in *E. coli,* PBP3 in *P. aeruginosa* is more resistant to MPO-derived oxidants, and PBPs 1a, 1b, 4, and 5 of *P. aeruginosa* are inactivated more rapidly than their counterparts in *E. coli.* Since correspondingly numbered PBPs in *E. coli* and *P. aeruginosa* are thought to have similar functions (25), the reason for this discrepancy in the pattern of MPO-mediated PBP inactivation is unclear. Different locations of critical, sensitive residues in the two organisms, with resulting differences in accessibility to MPO-derived oxidants, could explain this disparity.

P. aeruginosa PBPs 1a and 1b are thought to be critical for bacterial survival, and their inhibition by MPO-derived oxidants would be expected to lead to cell death, consistent with the good correlation seen between loss of PBP 1a and 1b activity and viability. Studies of *S. aureus* have suggested that PBP2 is the primary transpeptidase, PBP3 is involved in septation, and PBPs 1 and 4 are nonessential (26). Again, MPO-mediated inhibition of a critical PBP, in this case PBP2, would be expected to lead to cell death, and is consistent with the good correlation between loss of PBP2 activity and killing.

In summary, the penicillin binding capability of essential *E. coli, P. aeruginosa,* and *S. aureus* PBPs is lost in the presence of MPO-derived oxidants at rates approximating loss of microbial viability. MPO-derived oxidants presumably attack multiple bacterial targets simultaneously and the resultant oxidation events probably act in concert to effect loss of viability. The overall rapidity of PBP inactivation, the independent evidence from antibiotic studies demonstrating the lethality of PBP inactivation, and the suggestive morphologic changes in *E. coli* induced by MPO-derived oxidants all argue that PBP inactivation is a major mechanism of MPO-mediated microbicidal action. The extent to which PBP-inactivation is a feature of microbicidal activity mediated by intact neutrophils remains to be determined. However, preliminary studies have established that PBP activity of membranes from *E. coli* phagocytosed by human PMNs declines in parallel with microbial viability (unpublished observation). PBPs' vulnerability to inactivation appears to have been exploited by antimicrobial systems as diverse as mold-associated β-lactam antibiotics and mammalian phagocyte-associated MPO.

Acknowledgments

We thank Paul Mendelman and Donald Chaffin for assistance with penicillin-binding fluorographic methods and helpful discussions, Bryce Michel for technical assistance, and Marsha Faamuli for assistance in preparation of the manuscript.

This work was supported by grant AI 25606 from the National Institute of Allergy and Infectious Diseases.

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