BIOREDUCTION OF NITROXIDES BY STAPHYLOCOCCUS AUREUS

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Received September 7,1977

Summary

Two nitroxide radicals (TEMPO, I; OXAN, II) and a spin labeled penicillin (III) were reduced by Staphylococcus aureus. A short induction period preceded zero order reduction of these substrates leading to a K_m of 8×10^{-4}M, 6.67×10^{-5}M and 5.7×10^{-4}M and V_{max} of 106, 26 and 11 μ mole/min mg bacteria for I, II and III, respectively.

INTRODUCTION

The use of nitroxide radicals as reporter groups for probing macromolecular structure has been well documented (1). We prepared a spin labeled penicillin (III) in order to observe its interaction with transpeptidase, in vivo using electron paramagnetic resonance techniques. Unfortunately, we found that the nitroxide moiety of the penicillin (III) was rapidly reduced by Staphylococcus aureus so that binding studies could not be performed.

We have previously reported the bioreduction of nitroxides by rat liver microsomes and have attributed this reduction to cytochrome P-450 (2). Although Staphylococcus aureus has not been reported to contain cytochrome P-450, it does contain cytochromes a, b, and o, with cythochrome o serving as the principle terminal oxidase, (3-5). Cytochrome o like cytochrome P-450 has been observed to form a CO complex (3). Staphylococcus aureus also contains an active nitrate reductase which has been shown to be coupled to the bacterial cytochrome system (6).

ISSN 0006-291X

Although we have not yet made an attempt to identify the enzyme(s) responsible for the nitroxide bioreduction, in this communication we will, present the results of our experiments using intact bacteria.

MATERIALS AND METHODS

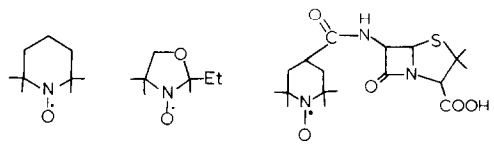
Staphylococcus aureus strain ATCC 25923 was obtained from Duke University Department of Microbiology and was grown aerobically at room temperature in Mueller-Hinton media. The bacteria were harvested by centrifugation at 2,500x g for 20 minutes during the late logarithmic growth phase. The bacterial pellets were washed twice and resuspended in 0.1 M Na_2HPO_4 buffered at pH 7.4 to obtain a final concentration of 14.8 mg dry bacteria/ml.

Preparation of 2,2,6,6-tetramethylpiperidine (TEMPO) I and 2-ethyl-2,4,4-trimethyl-3-oxazolidinyloxy (OXAN) II has previously been reported (7,8). The synthesis of the spin labeled penicillin (III) is outlined below. Mixing a tetrahydrofuran solution of ethylchloroformate with 1-oxyl-4-carbonyl-2,2,6,6-tetramethylpiperidine (9) gave the mixed anhydride. To this mixture, was added the triethylammonium salt of 6-aminopenicillanic acid (10) (Aldrich Chemical Company). Upon work-up, the spin labeled penicillin (III) was isolated.

Electron paramagnetic resonance spectra were obtained using a Varian Associates model E-9 spectrometer. The kinetic studies were conducted by measuring the decrease in the height of the central peak of the nitroxide triplet as a function of time. In a typical experiment, the reaction medium contained 1 x 10^{-4}M of the nitroxide, 0.2 ml of bacterial suspension and sufficient buffer (0.1M Na_2HPO_4, pH 7.4) to bring the final volume to 0.5 ml. These experiments were conducted at ambient temperature in a 0.1 mm flat cell. Bacterial activity of I, III, and penicillin G (Pfizer) were determined by the standard tube dilution technique (11).

RESULTS

The nitroxide moieties of TEMPO (I), OXAN (II) and SLP (III - spin labeled penicillin) were all reduced by Staphylococcus aureus as evidenced by the disappearance of the electron paramagnetic resonance signal upon incubation of the nitroxide with the bacteria. In all cases, incubation of the nitroxides with the bacteria exhibits an initial induction period followed by rapid reduction of the free radical. This induction varied from 81 to 142 sec. at 23°, with a mean of 103 and 119 sec. for TEMPO and OXAN, respectively. The induction period for SLP did not differ significantly from those for TEMPO and OXAN.



I TEMPO II OXAN III SLP

Preincubation of the microorganism in 3.3 mM benzyl penicillin solution for 30 min. at 23° did not inhibit the reduction of I or III. Likewise, 1 mM sodium azide was found not to inhibit these reductions. However heating the bacteria to 60° for 30 min. completely destroyed their nitroxide bioreductive activity.

The Michaelis-Menten constants K_m and V_{max} were determined using the Lineweaver-Burk procedure and are given in Table I.

The hydroxylamines of TEMPO and OXAN were not oxidized to their corresponding nitroxides upon incubation with the bacteria.

Bactericidal studies using the same strain of Staphylococcus aureus gave minimum inhibitory concentrations (MIC) of 128, 4, and .04 μg/ml for I, III, and benzyl penicillin, respectively.

DISCUSSION

Bacterial nitroxide reduction has not previously been reported. The nitroxyl reductase of Staphylococcus aureus appears to exist in abundance as evidenced by the rapid reduction of concentrated solutions of nitroxides and is probably an important enzyme in its metabolic scheme when grown in Mueller-Hinton meadia. However there appears to be no oxidase present to convert secondary hydroxylamines to nitroxide radicals.

We feel that the observed induction period represents nitroxide diffusion through the cell wall since the time interval is relatively short.

Table I

Michaelis-Menten Constants for Nitroxide Bio-reduction

Substrate	K_m	V_{max} *
TEMPO	8×10^{-4}M	106
OXAN	6.67×10^{-5}M	26
SLP	5.7×10^{-4}M	11

*The units for V_{max} are μmoles min^{-1} mg $bacteria^{-1}$.

TEMPO did not exhibit significant bactericidal activity. Thus, the bactericidal activity of SLP is undoubtedly related to its β-lactam ring and not to the nitroxide moiety.

The observation that benzyl penicillin does not inhibit the reduction of the spin labeled penicillin suggests that the site of bactericidal activity and reduction are not the same.

It is apparent that nitroxide spin labeled compounds are not satisfactory for use as spin resonance molecular probes in Staphylococcus aureus since reduction of the radical occurs prior to any observable binding. However, this reduction allows electron paramagnetic resonance spectroscopy to be used as a tool for kinetic measurements of nitroxide reduction. Nitroxides can be considered as oxygen analogs since they contain an unpaired electron and have a terminal oxygen. Hence, reaction with oxidoreductases are not unexpected.

Burke and Lascelles (6) have previously reported a nitrate reductase

in Staphylococcus aureus which is significantly inhibited by sodium azide. Since nitroxide reduction was not found to be antagonized by sodium azide, we believe that this nitroxyl reductase is an enzyme distinct from nitrate reductase. Further experiments will be directed towards identification and perhaps purification of the staphylococcal nitroxyl reductase.

ACKNOWLEDGEMENT

We would like to thank Lydia Tiosejo and Dr. Dolph Klein from Duke University Department of Microbiology for providing bacteria and facilities for our study.

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