Antibacterial Peptide from Normal Rabbit Serum. 3. Inhibition of Microbial Electron Transport†

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ABSTRACT: The influence of the primary rabbit serum bactericide, PC-III, on the respiratory activity of *Bacillus subtilis* has been examined. Glucose- or lactate-dependent respiration by whole cells was rapidly and completely inhibited by concentrations of the bactericide producing significant cell death. Similar results were observed with membrane vesicles oxidizing NADH. In both cases, bactericide-induced inhibition of respiration was calcium dependent and blocked electron transport between cytochromes *b* and *a*. PC-III competed with oxidized *Saccharomyces* cytochrome *c* when the latter was used as an electron acceptor in cytochrome *c* reductase reactions catalyzed by *B. subtilis* membrane vesicles. Competitive inhibition by PC-III was also observed when reduced *Saccharomyces* cytochrome *c* was used as electron donor in the cytochrome *c* oxidase reaction. At an ionic strength of 0.13, PC-III exhibits a k_i of 25.9 and 102 nM for the reductase and oxidase complexes, respectively. Increasing the ionic strength to that producing optimal antibacterial action against whole cells (0.24) increased the k_i of PC-III for the reductase (75.4 nM), while that for the oxidase decreased (92.3 nM).

The antibacterial and cytotoxic effects of many natural and synthetic polycationic compounds have been well documented [see Skarnes & Watson (1957)]. In particular, past observations on the action of protamines, histones (Miller et al., 1942), and basic tissue polypeptides (Watson & Bloom, 1952) have suggested primary lesions at the level of the cell membrane. More recent studies have revealed that significant alterations in cellular and mitochondrial processes occur in the presence of polycations. Of the functions for which effects have been demonstrated, including permeability (Zeya & Spitznagel, 1966), respiration (McEwen et al., 1963), and adenosine triphosphatase activity (Rosenthal and Buchanan, 1974), all are intimately associated with cytoplasmic membrane integrity (Harold, 1972). The ability of polycations to affect membrane electron transport and energy generation seems clearly established.

In addition to the general studies described above, Walton & Gladstone (1975) have demonstrated specific inhibition of NADH oxidase activity in membranes of staphylococci by cationic proteins. Additional experiments by Penniall et al. (1972) have shown similar effects on rat liver cytochrome *c* oxidase. The cationic proteins used in both studies were extracted from the lysosomes of rabbit polymorphonuclear leucocytes.

Synthetic polycations have also been shown to inhibit specific regions of the electron transport chain. In these studies, poly(L-lysine) has been used extensively and has been shown to inhibit both mitochondrial and bacterial cytochrome *c* reductase (complex III) and oxidase (complex IV) activities (Davies et al., 1972, 1963; Person et al., 1964; Smith et al., 1976). From the above data, it is the enzymatic activities of electron transport complexes which appear highly susceptible to the influences of polycations.

The studies described below demonstrate that the primary rabbit serum bactericide (PC-III),[1] a low molecular weight cationic peptide (Carroll & Martinez, 1981b), is a potent inhibitor of electron transport in whole cells and membrane vesicles of *Bacillus subtilis*. Furthermore, our results indicate that a possible primary action of the bactericide in vitro involves competitive inhibition with cytochrome *c* for binding sites on both the reductase and oxidase complexes. Under the specific ionic conditions found in normal serum, however, the peptide exhibits greater reactivity with the reductase. Some implications of these reactions as they pertain to nonspecific host defense, bactericide binding, and mechanism of action as well as the interactions of cytochrome *c* with respiratory components are discussed.

Experimental Procedures

Materials. Sodium azide, sodium dithionite, bovine serum albumin (BSA), DL-lactate, potassium ferricyanide, dinitrophenol (Dnp), and NADH were all obtained from Sigma. Hen egg white lysozyme (salt-free, Worthington Biochemicals), menadione (Mann Labs), and D-glucose (Nutritional Biochemicals Corp.) were obtained from the sources indicated. All other reagents were of analytical grade and were obtained from Mallinckrodt. The primary bactericide (PC-III) of normal rabbit serum was purified as described (Carroll & Martinez, 1981a). Concentrations of the peptide were determined fluorometrically, with molar values calculated by assuming a molecular weight of 2000 (Carroll & Martinez, 1981b).

Cytochrome c. *Saccharomyces cerevisiae* cytochrome *c* (type VIII, Sigma) was dissolved at 5 mM in 50 mM potassium phosphate buffer, pH 7.4 (phosphate buffer), rapidly frozen in small aliquots, and stored −70 °C. Oxidized cytochrome *c*, produced by treating an aliquot with minimal amounts of potassium ferricyanide, was chromatographed on a 0.8 × 25 cm column of Sephadex G-25 fine (Pharmacia) equilibrated in phosphate buffer at 4 °C. Fractions containing cytochrome *c* were pooled, divided into 0.2-mL aliquots, and rapidly frozen in a dry ice ethanol bath. Reduced cytochrome *c* was produced by a similar procedure by using minimal amounts of sodium dithionite. All samples were stored at −70

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[1] Abbreviations used: PC-III, biologically active, antibacterial rabbit serum fraction eluting from phosphocellulose [see Carroll & Martinez (1981a)]; BSA, bovine serum albumin; Dnp, dinitrophenol.

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°C and thawed immediately before use. Stored samples remained greater than 97% oxidized or reduced, respectively. Cytochrome *c* concentrations were calculated following dithionite reduction by using millimolar extinction coefficients (ϵ_{mM}) of 29.0 at 550 nm (Yonetani, 1965) or 140.5 at 411.5 nm (see text).

Preparation of Cells and Membrane Vesicles. Unless indicated otherwise, growth conditions for the test organism, *Bacillus subtilis* 168 (*trp*–, *fla*–), were as previously described (Carroll & Martinez, 1979). Membrane vesicles were isolated essentially as described by Konings et al. (1973) by using phosphate buffer for all incubations. Following lysis and vesicle formation, the membranes from 10^{12} cells were resuspended in 50 mL of cold phosphate buffer containing 1.0 mM $MgCl_2$ by using a Potter–Elvehjem homogenizer. The vesicle preparation was centrifuged at 800*g* for 30 min at 4 °C to remove any remaining cellular debris, and then the membranes were harvested by centrifugation (20000*g*, 30 min, 4 °C). The sedimented membranes were washed once with phosphate buffer containing 1.0 mM $MgCl_2$, washed once with phosphate buffer alone, and then resuspended in 10 mL of phosphate buffer by brief homogenization. Aliquots of 0.2 and 0.4 mL were dispensed in small plastic tubes, rapidly frozen in a dry ice ethanol bath, and stored at −70 °C. These vesicle preparations were thawed and diluted immediately prior to analysis. Under these conditions, membrane-associated enzyme activities were stable for several weeks. Protein concentrations were determined by the method of Lowry et al. (1951).

Measurements of Oxygen Consumption and Lysis. Oxygen consumption by whole cells or membrane vesicles was examined in a Gilson K-IC Oxygraph equipped with a Yellow Springs Instruments Clark-type electrode. Reaction vessels were thermostated to 37 °C and contained a final volume of 2.0 mL. Whole cells, cultivated in glucose minimal medium to mid log phase, were harvested and resuspended to 2×10^8 cells/mL in cold phosphate buffer containing either glucose or lactate as indicated in the text. Reaction mixtures contained 5×10^7 cells/mL in phosphate buffer, 0.1% bovine serum albumin, and 1% homologous oxidizable substrate. The final ionic strength was adjusted to 0.24 with 1.5 M NaCl, with $CaCl_2$ and PC-III additions as indicated in the text.

Membrane vesicle respiration was examined under similar conditions to those described for whole cells. Phosphate buffer, NaCl, and samples of the vesicle preparations (final concentration of membrane protein 40 μg/mL) were placed in the thermostated vessel, and endogenous respiration was monitored for several minutes. This rate was usually negligible compared to that observed in presence of substrate. Respiration was initiated by the addition of NADH to a final concentration of 2.5 mM. Additions of $CaCl_2$ and/or PC-III were made at the times and concentrations indicated in the text and figure legends.

Lysis of whole cells or membrane vesicles in response to PC-III action was continuously monitored at 540 nm in a Beckman Model 24 recording spectrophotometer thermostated to 37 °C. Whole cells were treated as for the bactericidal assay (Carroll & Martinez, 1979) and examined at 2×10^7 cells/mL. Membrane vesicles were examined at final protein concentrations of up to 0.2 mg/mL.

Cytochrome Content of Membranes and Whole Cells. The dithionite-reduced minus oxidized or NADH-reduced minus oxidized cytochrome absorption spectra were examined at a band-pass of 1 nm by using an Aminco DW-2a spectrophotometer (sample volume 2.5 mL). Vesicle preparations were diluted in phosphate buffer and examined at a final protein concentration of 1 mg/mL. After a base line was established at 25 °C, the samples were reduced with either minimal sodium dithionite or 1 mM NADH and scanned between 400 and 650 nm. Whole cells were cultivated as described, washed 2 times, and resuspended in phosphate buffer to 10^{10} cells/mL. The reference cells were oxidized by bubbling oxygen through the cuvette for 5 min, while the sample was reduced with minimal amounts of dithionite.

Temporal effects of PC-III action on the oxidation–reduction state of membrane respiratory components were examined by analysis of changes in the characteristic Soret absorption spectra. Vesicle preparations (0.2 mg/mL) in 10 mM phosphate buffer wer reduced by the addition of 0.5 mM NADH and then divided into reference and sample cuvettes. After several minutes, the sample cuvette received calcium to a final concentration of 0.8 mM, followed by 100 μL of PC-III (final concentration 4 μg/mL) in 1.0 M phosphate buffer. The reference cuvette received identical concentrations of $CaCl_2$ and 1.0 M phosphate buffer. Final concentration of phosphate in both reaction mixtures was 50 mM. Alterations in steady-state concentrations of reduced and oxidized forms of cytochromes *a* and *b* were examined as a function of time at 440 and 424 nm, respectively (see text).

NADH:Cytochrome c Oxidoreductase Activity. The NADH-cytochrome *c* reductase activity (respiratory complex I–III) catalyzed by membrane vesicles was examined spectrophotometrically by using an Aminco DW-2a spectrophotometer in the dual-beam mode. Two separate wavelength pairs were used for analyses, depending on the concentration of cytochrome *c* used. Reaction mixtures contained 41 μg of membrane protein/mL, 1.0 mM $CaCl_2$, and sufficient 1.0 M phosphate buffer such that, after addition of PC-III (stored in 1.0 M phosphate), the final phosphate concentration was 50 mM. NaCl was added as indicated. The membranes were preincubated for 5 min in the presence or absence of the bactericide, followed by the additon of NaN_3 to 4.0 mM, NADH to 100 μM, and oxidized cytochrome *c* (0.07–2.0 μM). For concentrations between 0.5 and 2.0 μM, the rates of cytochrome *c* reduction were monitored at 545.8 nm − 530 nm, with calculations based on an ϵ_{mM} of 23.9. Reactions containing lower concentrations of cytochrome *c* (0.07–0.5 μM) were monitored at 411.5 nm − 397.5 nm by using a calculated ϵ_{mM} of 71.1 (see text). For the latter reactions, the spectrophotometer was first set to the appropriate wavelengths, and then the beam balance was adjusted to zero on the 0.1 *A* scale in the presence of oxidized cytochrome *c*. Following the addition of NADH, but prior to the addition of membranes, the rates of nonenzymatic reduction of cytochrome *c* were monitored for 2 min at each concentration examined. The values obtained were used to calculate specific reduction rates in the remaining assays. Under these conditions, the first 1–2 min of the reaction were usually linear, allowing accurate quantitation of the initial steady-state rates of cytochrome *c* reduction. In the range of enzyme and cytochrome *c* concentrations used in the inhibition studies, reaction rates were first order with respect to the cytochrome *c* concentration. Results are presented as nanomoles of cytochrome *c* reduced per minute per milligram of membrane protein. A similar method for the analysis of beef mitochondrial cytochrome *c* reductase activity was recently described by Speck et al. (1979).

Ferrocytochrome c:Oxygen Oxidoreductase Activity. Cytochrome *c* oxidase activity (complex IV) was assayed by methods similar to those described for the reductase. Reaction mixtures contained 41 μg of membrane protein/mL and 1 mM

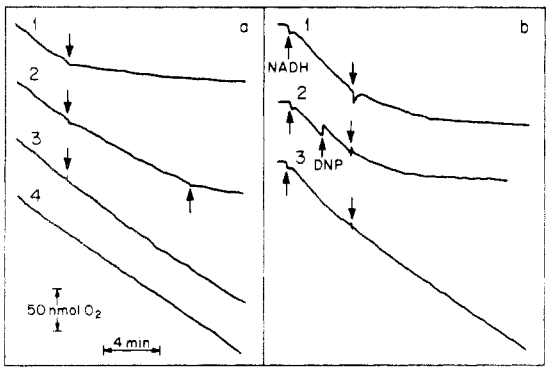

FIGURE 1: Effects of PC-III on respiration rates in whole cells (a) and membrane vesicles (b) of *B. subtilis*. (a) Whole cells were examined at a final concentration of 5×10^7/mL in 2 mL of 50 mM phosphate buffer (pH 7.4) containing 1% glucose, 0.1% BSA, and NaCl to an ionic strength of 0.24. Endogenous respiration rates at 37 °C were monitored by using an oxygen electrode for several minutes prior to the addition of samples (at the arrows). Additions were as follows: (Tracing 1) both PC-III (100 ng, 25 nM) and $CaCl_2$ (final concentration 1.0 mM) were added. (Tracing 2) Following a 4-min preincubation of the cells with 100 ng of PC-III (before the first arrow), $CaCl_2$ was added to 1.0 mM. Nine minutes later (second arrow), a second addition of PC-III (100 ng) was made. (tracing 3) A 100-ng sample of PC-III alone was added. (Tracing 4) No additions were made. (b) Membrane vesicles were examined at a final protein concentration of 40 μg/mL in phosphate buffer (ionic strength 0.24). At the first arrows, NADH was added to 2.5 mM, and an endogenous respiration rate was established. Additions were as follows: (tracing 1) 100 ng of PC-III and $CaCl_2$ to 1.0 mM; (tracing 2) Dnp (dissolved in ethanol) to 0.5 mM, followed 3 min later by both PC-III (100 ng) and $CaCl_2$ (to 1.0 mM); (tracing 3) 100 ng of PC-III alone.

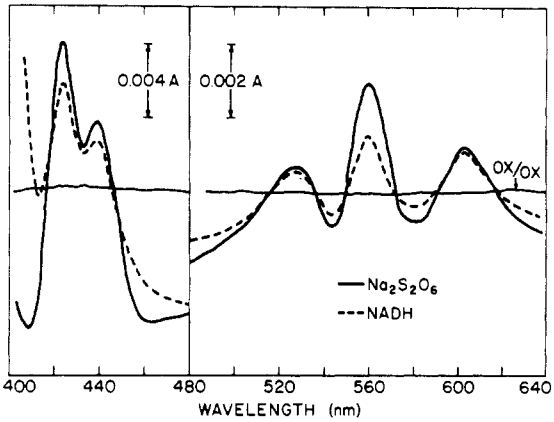

FIGURE 2: Difference spectra of membrane vesicles of *B. subtilis*. Samples were examined at room temperature (25 °C) in an Aminco DW-2a spectrophotometer using a band-pass of 1 nm. The vesicles were resuspended in 50 mM phosphate buffer (pH 7.4) to 1 mg of membrane protein/mL (5 mL total volume) and divided into the reference and sample cuvettes. Following the establishment of an ox/ox base line, the sample was reduced with minimal sodium dithionate or NADH (to 1.0 mM) and the spectrum scanned 1–2 min later.

$CaCl_2$ in 50 mM phosphate buffer. Following the addition of reduced cytochrome *c* (final concentration 0.07–2.0 μM), a basal rate of cytochrome *c* oxidation at 25 °C was established. The reaction was initiated by adding the membrane preparation to a final protein concentration of 41 μg/mL. Prior to the analysis of reaction rates using concentrations of cytochrome *c* between 0.07 and 0.5 μM, the beam intensities at 411.5 and 397.5 nm were adjusted to zero for sample containing 0.5 μM oxidized cytochrome *c*. For the analysis of bactericide action, the factor was preincubated with the membranes for 5 min at the concentrations and ionic strength indicated in the text and figure legends prior to the addition of reduced cytochrome *c*. Steady-state reaction rates (expressed in terms of nanomoles of cytochrome *c* oxidized per minute per milligram of membrane protein) were calculated from the initial linear regions of the recorder tracings.

Results

Action of PC-III on Whole Cells and Membranes of B. subtilis. The effect of PC-III on respiration by whole cells and membranes at an ionic strength of 0.24 is shown in Figure 1. For these experiments, reaction mixtures contained concentrations of the bactericide (50 ng/mL, 25 nM) which exhibit potent antibacterial action (ca. a 4–5 log kill within 20 min) (Carroll & Martinez, 1981a). It is clear (Figure 1a) that addition of PC-III and calcium to whole cells actively respiring on glucose rapidly, if not immediately, abolished oxygen consumption. Preincubating whole cells with PC-III in the absence of $CaCl_2$ did not alter the respiratory rate. Further, the subsequent addition of calcium did not result in significant reductions of oxygen consumption. A second addition of an identical concentration of PC-III 9 min later rapidly reduced the rate of respiration. As expected, adding PC-III to cells actively respiring in the abscence of calcium did not affect respiration rates when compared to whole cells alone. Although respiration rates were somewhat higher when the ionic strength was reduced to 0.13, they were equally susceptible to inhibition by PC-III. Similar results were obtained when reactions contained cells respiring on lactate (data not shown).

Respiration rates of membrane vesicles (40 μg/mL) oxidizing NADH were also sensitive to PC-III (Figure 1b). In contrast to the rapid effects observed with whole cells, however, addition of the bactericide (50 ng/mL, 25 nM) and $CaCl_2$ to respiring vesicles did not result in an immediate cessation of oxygen consumption. Rather, a transition period of ~6 min was observed during which respiration rates continuously decreased. Although increasing the concentration of PC-III from 10 to 100 ng/mL reduced the length of the transition, immediate inhibition of O_2 uptake was never achieved (data not shown). Identical results were obtained in the presence of 0.5 mM DNP; i.e., inhibition of oxygen consumption occurred but required several minutes for maximal expression. These observations may reflect the orientation of the membrane vesicles (Hampton & Freese, 1974). As with whole cells, PC-III-induced reductions in respiration were calcium dependent.

The effects of PC-III on whole cells or membranes were not due to gross lysis of the cells or vesicle preparations. This was shown turbidometrically by using a range of vesicle, cell, and PC-III concentrations in the presence and absence of calcium with continuous monitoring at 540 nm. Under these conditions (data not shown), the turbidity of cell suspensions remained stable for at least 30 min, while that of the vesicles was unaffected for several hours.

Cytochrome Content of B. subtilis. The above results demonstrated that PC-III effectively blocked microbial respiration at concentrations exhibiting potent antibacterial action. Moreover, the site of action appeared to be located within the respiratory complex involved in passing electrons from NADH to oxygen.

Membrane preparations of *B. subtilis* contain cytochromes *a*, *b*, *c*, and c_1 (Miki et al., 1967). The dithonite-reduced minus oxidized and NADH-reduced minus oxidized visible absorption spectra of a membrane vesicle preparation (final protein concentration 1 mg/mL) (Figure 2) suggested that similar respiratory components were present in strain 168. Tentative identification of these maxima was as follows (Konings & Freese, 1972; Miki et al., 1967): bands at 603 and 440 nm for cytochrome *a*; bands at 560, 528, and 424 nm indicative of

Table I: Alterations in the Oxidation-Reduction State of Steady-State-Reduced Membrane Vesicles of *B. subtilis* in Response to PC-III[a]

addition to vesicles	ΔA ($\times 10^3$) *b* type (424 nm)	*a* type (440 nm)
NADH	0	−0.05
NADH + $CaCl_2$	−0.10	+0.07
NADH + PC-III	+0.08	0
NADH + $CaCl_2$ + PC-III	+2.02	−0.53

[a] Reaction mixtures (4.8 mL) contained membrane vesicles (final concentration 0.2 mg of protein/mL) and 1.0 mM $CaCl_2$ in 10 mM phosphate buffer (pH 7.4). Following the addition of NADH to 0.1 mM, the mixtures were divided into reference and sample cuvettes and placed in the spectrophometer (1-nm band-pass), and steady-state rates were achieved. After 2 min, PC-III was added to 4 μg/mL (final concentration of phosphate 50 mM), and the difference spectra at 424 and 440 nm for reduced cytochromes *b* and *a*, respectively, were recorded. Values presented above were calculated directly from the recorder tracing 2 min after the addition of PC-III.

cytochrome *b*. Although not readily apparent in Figure 2, a slight shoulder on the cytochrome *b* 560-nm peak was routinely observed, suggesting the presence of low concentrations of cytochrome c. This result is consistent with data concerning changes in *B. subtilis* respiratory chain components during vegetative growth (Tochikubo, 1971; Taber, 1974). Exposure of the membrane vesicles to 1.0 mM NADH resulted in only partial reduction of all cytochromes (Figure 2). The absorption spectra between 460 and 510 nm also indicated incomplete reduction of flavoproteins.

Dithionite-reduced whole cell suspensions exhibited similar spectra to those described for the membrane vesicles (data not shown). Calculation of the cytochrome *a* content in both the cell and vesicle preparations on the basis of the reduced minus oxidized absorption spectra (Δ603–630 nm) indicated that 1 μg of membrane protein had the equivalent cytochrome content of 10^6 cells.

Action of PC-III on Respiratory Components. Effects of the bactericide on membrane respiratory components were examined as a function of time by using the absorption maxima described above. Prior to PC-III addition, aerobic steady-state oxidation rates were established in both the sample and reference cuvettes by the addition of 0.1 mM NADH. Alterations in the difference spectra would thus be indicative of the respiratory oxidation–reduction state. Initially, attempts were made to examine the characteristic cytochrome α absorption spectra by rapid repeat scanning from 550 to 620 nm. The vesicles were examined at 0.2 mg protein/mL to maintain the ratio of bactericide to membranes similar to that used in the respiration experiments above. Additions of PC-III, stored in 1.0 M phosphate, were made such that the final concentrations of phosphate and ionic strength were 50 mM and 0.13, respectively. Due to the low cytochrome levels in the vesicles at the concentrations used, it was difficult to correlate slight variations in cytochrome α spectra as a function of PC-III action.

Analysis of the Soret absorption spectra by the same techniques indicated that concentrations of reduced cytochrome *b* increased, while that of cytochrome *a* decreased, following addition of PC-III (4 μg/mL) (data not shown). These results were illustrated more clearly by difference spectral analysis of the individual Soret maxima following incubation of several minutes wth PC-III (Table I). Calcium-dependent increases in both reduced cytochrome *b* and oxidized cytochrome *a* were observed immediately following bactericide addition. The

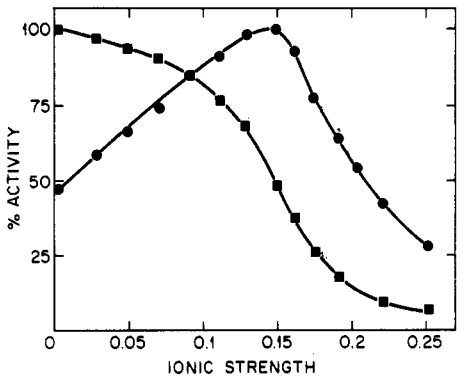

FIGURE 3: Effects of ionic strength on cytochrome *c* reductase (●) and oxidase (■) activities of *B. subtilis* membrane vesicles. All reaction mixtures contained 27.4 μg of membrane protein/mL, 0.38 μM *Saccharomyces* cytochrome *c*, and 1.0 mM $CaCl_2$ and were examined spectrophotometrically in an Aminco DW-2a spetrophotometer in the dual-beam mode at 25 °C. The wavelength pair used in these analyses was 545.8–530 nm. Ionic strength below 0.13 were adjusted by varying the final concentration of phosphate, while those above 0.13 (50 mM phosphate buffer plus $CaCl_2$) were increased by the addition of NaCl. Reductase activities were examined in the presence of 100 μM NADH and 4.0 mM sodium azide. From the linear portions of the time course, rates were calculated in terms of nmoles cytochrome *c* reduced (or oxidized) per minute per milligram of membrane protein. Results are presented as percent maximal activity observed for each enzyme.

passage of electrons from cytochrome *b* (respiratory complex III) to cytochrome *a* (complex IV) was thus rapidly inhibited by the bactericide.

Analysis of Terminal Respiratory Components. Before the effects of PC-III on terminal electron transfer complexes could be examined, it was necessary to establish reaction conditions for the vesicle preparation. Mammalian cytochromes *c* are not suitable substrates for cytochrome *c* oxidase activity catalyzed by *B. subtilis* membrane preparations (Miki & Okunuki, 1969; Smith, 1954). *Saccharomyces* cytochrome *c*, which reacts quite rapidly with the bacterial oxidase (Miki & Okunuki, 1969; Miki et al., 1967), was therefore studied. Reduced and oxidized forms of the yeast cytochrome *c* were first scanned between 350 and 650 nm. The reduced form in phosphate buffer exhibited α, β, and Soret peaks at 545.8, 516.5, and 411.5 nm, respectively. When an ϵ_{mM} of 29.0 was used for the reduced cytochrome at 550 nm (Yonetani, 1965), extinction coefficients of 23.9 and 71.1 were calculated for the wavelength pairs 545.8–530 and 411.5–397.5 nm, respectively. The latter pair bisects the oxidized Soret peak (404.5 nm) and allowed spectral analysis of cytochrome *c* concentrations as low as 0.07 μM. The presence of NADH in these reactions was found to influence the Soret absorption spectra. Control incubations in the absence of membranes adequately quantitated these background effects as well as the endogenous cytochrome *c* reduction rates produced by NADH. When examined in this manner, the reductase activity was inhibited by 0.1 mM rotenone or antimycin A, while oxidase activity was blocked in the presence of 4.0 mM azide.

The rates of both oxidase and reductase reactions in the presence of calcium were markedly influenced by ionic strength (Figure 3). Whereas the reductase activity exhibited a maximum at 0.15, the activity of the oxidase continuously decreased above 0.004. The percentage of maximal activity observed at an ionic strength of 0.13 was 99% and 65% for the reductase and oxidase, respectively. At the ionic strength producing maximal activity of the bactericide on whole cells (0.24), reductase and oxidase activities were reduced to 32% and 7%, respectively. Increasing the ionic strength from 0.13 to 0.24 thus significantly decreased the reductase (3-fold) and

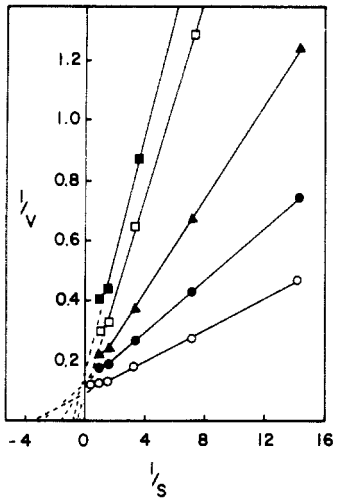

FIGURE 4: Inhibition of *B. subtilis* cytochrome *c* reductase activity by PC-III. Reactions were performed in 50 mM phosphate buffer (pH 7.4) containing 41 μg of membrane protein/mL, 1.0 mM $CaCl_2$, and NaCl as described below. After a 5-min preincubation of the membranes in the presence or absence of PC-III, sodium azide was added to 4.0 mM, followed by *Saccharomyces* cytochrome *c* (0.07–2.0 μM) and NADH (100 μM). Depending upon the concentration of cytochrome *c* used, reactions were examined at the wavelength pairs 545.8–530 (α peak) or 411.5–397.5 nm (Soret) as described in the text. The concentrations of PC-III examined at an ionic strength of 0.13 were 15 [30 ng/mL (●)] or 30 nM [60 ng/mL (▲)] and were compared to incubations in the absence of PC-III (○). At the higher ionic strength (0.24), the incubations were as follows: (□) buffer alone; (■) 15 nM PC-III (30 ng/mL). Data are presented as a Lineweaver–Burk double-reciprocal plot, with velocity (*V*) (corrected for nonenzymatic reduction of cytochrome *c*) expressed in terms of nmoles of cytochrome *c* reduced per minute per milligram of membrane protein and *S* in μM.

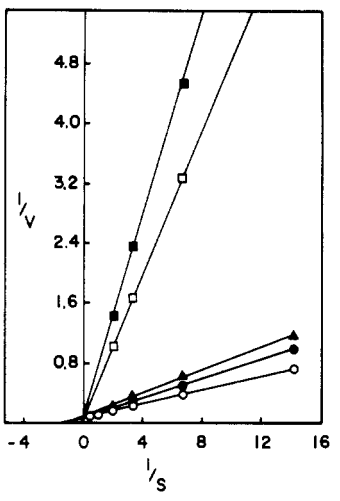

FIGURE 5: Inhibition *B. subtilis* cytochrome *c* oxidase activity by PC-III. Reactions were performed in 50 mM phosphate buffer (pH 7.4) containing 41 μg of membrane protein/mL and 1.0 mM $CaCl_2$. Following a 5-min preincubation in the presence or absence of PC-III, *Saccharomyces* cytochrome *c* was added to a final concentration between 0.07 and 2.0 μM. Initial reaction rates were then monitored at 545.8–530 or 411.5–397.5 nm, as indicated in the text. For the lower ionic strength reactions (0.13): (○) no inhibitor; (●) 30 nM PC-III (60 ng/mL); (▲) 150 nM PC-III (300 ng/mL). At an ionic strength of 0.24: (□) no inhibitor; (■) 30 nM PC-III (60 ng/mL). Data are presented as a Lineweaver–Burk double-reciprocal plot, with *V* expressed in terms of nmoles of cytochrome *c* oxidized per minute per milligram of membrane protein.

oxidase (9-fold) activities and did so in a competitive manner (data not shown).

Action of PC-III on Cytochrome c Reductase. The reaction kinetics of oxidized cytochrome *c* with the membrane vesicle preparations were examined in the presence and absence of PC-III. Reactions were performed in 50 mM phosphate buffer containing 1 mM $CaCl_2$ (final ionic strength 0.13). Eadie–Hofstee single reciprocal plot analysis of the data indicated that in the absence of the bactericide, the reaction of cytochrome *c* with the reductase had an apparent K_m of 0.27 μM. Deviations from linearity were observed, however, at cytochrome *c* concentrations above 0.6 μM. Adding increasing concentrations of PC-III (15–30 nM) to reaction mixtures rapidly inhibited the rates of reduction. This inhibition appeared to be competitive in nature with cytochrome *c* and did not occur in the absence of calcium ions. In these reactions, breaks from linear behavior above 0.6 μM cytochrome *c* were also observed. These deviations from Michaelis–Menten kinetics most probably reflect conversion of the first-order reaction at lower substrate concentrations to zero order at concentrations of cytochrome *c* above 0.6 μM (Errede & Kamen, 1978; Smith et al., 1974). Thus, at the constant concentraions of enzyme and NADH employed, some other reaction between NADH and cytochrome *c* becomes rate limiting at the higher substrate concentrations. For all subsequent analyses, rates were examined at cytochrome *c* concentrations below 1 μM to ensure first-order kinetics.

The inhibition of cytochrome *c* reductase produced by PC-III is shown more clearly in a Lineweaver–Burk double-reciprocal plot (Figure 4). The rabbit serum bactericide inhibited the reaction of cytochrome *c* with its reductase in essentially a competitive manner. At the highest concentration of PC-III examined (60 ng/nL, 30 nM), the apparent K_m of the reaction was increased by a factor of 6 (1.54 μM). From the slopes of these plots (ionic strength 0.13), a K_i of 25.9 nM was calculated for PC-III. Increasing the ionic strength to 0.24 increased the K_i to 75.4 nM. Omitting calcium ions resulted in no inhibition. No evidence was found for the direct interaction of PC-III with isolated horse heart or *Saccharomyces* cytochromes *c*.

Action of PC-III on *Cytochrome c Oxidase.* The ability of the bactericide to compete with cytochrome *c* for the reductase suggested that a similar effect might occur with the oxidase. Although PC-III did in fact competively inhibit oxidase activity (Figure 5), the effect was much reduced compared to that on the reductase. At the highest concentration examined (300 ng/mL, 150 nM), the K_m of the reaction was increased by less than a factor of 2 (0.52–0.97 μM). The K_i at this ionic strength (0.13) was 102 nM, nearly 5 times greater than that for the reductase. In contrast to the situation with the reductase, increasing the ionic strength to that which allowed maximal expression of bactericidal activity against whole cells (0.24) decreased the K_i to 92.3 nM, a 10% reduction. Clearly, increasing the ionic strength had only a slight effect on the bactericide-induced inhibition of cytochrome *c* oxidase.

Discussion

Previous studies with PC-III (Carroll & Martinez, 1981a,b) have demonstrated its potent antibacterial action at concentrations above 2 ng/mL, with maximal activity being observed at an ionic strength of 0.24. The results presented here demonstrate that under identical conditions, the bactericide effectively blocks respiration in whole cells and membrane vesicles of *B. subtilis*. This inhibition, instantaneous with whole cells but exhibiting a short lag period with membranes, results from a specific lesion within the electron transport chain, one which inhibits the transfer of electrons from cytochrome *b* (complex III) to cytochrome *a* (complex IV). As with the microbicidal action of PC-III, its ability to inhibit respiration

at the concentrations examined is dependent on divalent cations.

Competitive inhibition between PC-III and *Saccharomyces* cytochrome *c* is clearly indicated by our data, suggesting that the antibacterical nature of the peptide may reside in its affinity for specific binding sites on respiratory chain components. This is apparent from the low inhibitor cnstants calculated from reaction rates in the presence of increasing inhibitor concentrations. At roughly the ionic strength of normal serum (0.13), the bactericide exhibits K_i of 25.9 and 102 nM for the reductase and oxidase enzymes, respectively. The peptide is thus more effective at inhibiting the reductase under physiological conditions.

That PC-III may also influence the reductase reaction in something other than a competitive manner is suggested by the variations in V_{max} observed at different concentrations of the peptide (Figures 4 and 5). It must be emphasized, however, that the reductase was analyzed as part of a membrane complex (I-III) containing not only the reductase but also NADH oxidase. As such, variations in the steady-state concentration of reduced ubiquinone in response to PC-III action would be expected to alter reductase kinetics. Analysis of the NADH oxidase complex using menadione as electron acceptor according to Hatefi (1978) (data not presented) indicated that activity was stimulated in the presence of the bactericide and may account for the deviations observed. Rosenthal & Buchanan (1974) have also noted a stimulation of *B. subtilis* ATPase activity in response to several cationic bactericides, including a partially purified preparation from rabbit serum. Thus, although certain cationic bactericides may exhibit primary sites of action, nonspecific interactions with the membrane (or associated membrane components) may contribute to their lethal effects.

Additional support for this contension comes from the studies of Amano and co-workers concerning the action of *plakin*, an antibacterial preparation obtained from horse and rabbit platelets (Amano et al., 1953). Their data indicate that although plakin inhibits respiration in whole cells (Amano et al., 1958), it also possesses phospholipase A activity capable of disrupting cellular membranes (Higashi et al., 1963, 1966). In contrast, we have been unable to detect any such activity in PC-III preparations; whole cells or membrane vesicles were not lysed by PC-III action, nor were a variety of erythrocyte suspensions (data not shown). If PC-III is in fact derived from plakin as suggested (Carroll & Martinez, 1981a), then the enzymatic and respiratory-inhibiting functions represent distinct domains on the plakin molecule.

Polycation inhibition of respiration or cytochrome *c* oxidase activity is known to be influenced by several physical parameters. These include (i) the density of basic residues, (ii) the density of hydrophobic residues, (iii) total molecular weight, and (iv) the ionic strength at which the reaction is performed (Katchalski et al., 1952; Person et al., 1964; Watson & Bloom, 1952). The influence of hydrophobic residues within mixed polycation polymers becomes increasingly apparent above ionic strengths of 0.1, where they are required for maximal inhibition (Person et al., 1964). The interactions of eukaryotic cytochrome *c* (itself a basic protein) with its oxidase (Furguson-Miller et al., 1976; Smith et al., 1977) and reductase (Ahmed et al., 1978; Speck et al., 1979) complexes, reactions markedly influenced by slight modifications of specific lysine residues, also appear to be both electrostatic and hydrophobic (Person et al., 1964). The ability of PC-III to compete with cytochrome *c* under physiological conditions may involve similar interactions.

Indeed, previous observations (Carroll & Martinez, 1981a), combined with decreased K_i for the oxidase at higher ionic strength, implicate the importance of the nonpolar residues (35 mol %) in the bactericidal reaction. Within this context, it is also of interest that of the two lysine residues present in the active peptide, one appears to be N^{ϵ}-monomethyl substituted (Carroll & Martinez, 1980b). It has been observed (DeLange et al., 1969, 1970; Dickerson, 1978; Dickerson & Timkovich, 1975) that many cytochromes *c* of eukaryotic microorganisms (including *Saccharomyces*) and higher plants contain at least one N^{ϵ}-trimethyllysine, usually located at position 72 (based on the numbering system for mammalian cytochromes) (Margoliash & Schejter, 1966). Although methyllysines also occur in histones (Hempel et al., 1968; Hnilica, 1967), they have not been found in any animal or bacterial *c* cytochromes (Dickerson, 1978; Dickerson & Timkovich, 1975). Whether this similarity between *Saccharomyces* cytochrome *c* and PC-III, i.e., the presence of methylated residues, is important in PC-III action has yet to be established.

The above studies describe a biochemical action of the rabbit serum bactericide, PC-III, and are consistent with the presumed orientation of respiratory components within the bacterial membrane (Harold, 1972). We are not yet convinced, however, that the inhibition of oxygen consumption as described represents the lethal event. When *B. subtilis* is growing on glucose, it derives most of its energy from substrate level phosphorylation (Szulmajster & Hanson, 1965), suggesting that interruption of oxidative phosphorylation should have little effect on cellular energetics. In all our reactions, glucose-grown cells were extremely susceptible to the action of PC-III. However, since the terminal respiratory components appear linked to the production of a proton gradient across the cytoplasmic membrane (Chance et al., 1977; Moyle & Mitchell, 1978; Papa, 1976; Wikstrom & Krab, 1978), an alternate possibility may be that PC-III-induced inhibition of electron transport reduces the cells ability to generate a transmembrane potential. This and other possibilities as well as the action of PC-III on mitochondrial preparations are currently under investigation. It is anticipated that characterization of the structure and sequence of this bactericide may provide further insight into its action and microbicidal specificity, defining its potential experimental and therapeutic use.

Acknowledgments

We express our gratitude to June Lascelles for valuable discussions throughout the course of this work as well as the generous loan of laboratory equipment.

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