INHIBITION OF THE RESPIRATORY-LINKED MEMBRANE POTENTIAL IN *E. COLI* MEMBRANE VESICLES BY OCTAPEPTIN

PAUL E. SWANSON and DANIEL R. STORM

Department of Pharmacology, University of Washington,
Seattle, Washington 98195, U.S.A.

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Octapeptin is a peptide antibiotic which affects bacterial membrane structure and selective membrane permeability for protons and potassium. The influence of octapeptin on the formation of a membrane potential generated across bacterial vesicles was monitored using the Rb^+-valinomycin transport system. Octapeptin inhibited the respiratory-linked generation of membrane potentials formed in the presence of succinate or Asc/PMS. In addition, the antibiotic inhibited [^{3}H]-leucine transport driven either by succinate or Asc/PMS. These studies support the proposal that the antimicrobial activity of octapeptin is due to inhibition of the formation of a membrane potential generated in the presence of appropriate respiratory substrates.

The octapeptins are a closely related class of peptide antibiotics[1] which exhibit a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria[2]. The octapeptins are structurally related to the polymyxins and are characterized by a cyclic octapeptide containing a C: 10 or C: 11 β-hydroxy fatty acid attached to the peptide through an amide bond[3,4,5].

A number of biochemical processes in bacteria are affected by octapeptins but mechanistic studies have strongly implicated the bacterial membrane as the primary site of action[6]. Previous studies have indicated that the effects of octapeptin on bacterial metabolism are similar to those of uncouplers of oxidative phosphorylation[7]. The antibiotic stimulated bacterial respiration at concentrations corresponding to minimum inhibitory concentrations. It also enhanced the membrane proton permeability and lowered the adenosine 5′-triphosphate pool size. It was proposed that octapeptins disrupt the selective ion permeability of the cytoplasmic membrane, thereby relaxing the membrane potential. In the present study, this hypothesis was directly tested by examining the influence of octapeptin on the membrane potential in *E. coli* membrane vesicles. It is demonstrated that octapeptin inhibits both formation of the membrane potential generated in the presence of respiratory substrates and respiratory coupled leucine transport.

Materials and Methods

Materials

Octapeptin (EM49)·4HCl was obtained from the Squibb Institute for Medical Research. Valinomycin, CCCP, sodium ascorbate, and sodium succinate were obtained from Sigma. PMS was obtained from Aldrich, sodium azide from Eastman, L-leucine from ICN, and RbCl from Apache Chemicals. [$^3H_3$]-L-Leucine (116 Ci/mmole) and [$^{86}Rb^+$]Cl^- (364 mCi/mmole) were obtained from New England Nuclear. 25 mM Cellulose acetate filters (0.45 μ) were purchased from Curtis Matheson Scientific, Inc.

* Abbreviations: Asc/PMS, phenazine methosulfate reduced by ascorbate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; MIC, minimum inhibitory concentration.

Methods

Growth of Bacteria and Preparation of Vesicles

Escherichia coli strain ML-308-225 was grown on Minimal Medium A[8] containing 1% sodium succinate as a carbon source and a trace of $FeSO_4 \cdot 7H_2O$ (0.00005%). Membrane vesicles were prepared as described[9], and 1 ml aliquots were frozen and stored in liquid N_2 at 4.5 mg protein/ml in 100 mM potassium phosphate at pH 6.6.

Transport Assays

In all cases, frozen vesicles were thawed rapidly at 46°C. For [$^3H_3$]-leu transport assays, the vesicles were diluted to 1 mg protein/ml in 100 mM potassium phosphate, pH 6.6, incubated at 25°C for 20 minutes and stored on ice until assayed. All transport assays were done in buffers containing no Mg^{2+} since it inhibits the antimicrobial activity of octapeptin[11]. Mg^{2+} is not required for the coupling of respiration to either leu transport or valinomycin facilitated Rb^+ transport. For $^{86}Rb^+$ uptake, the vesicles were treated as previously described[10] using 100 mM sodium phosphate, pH 6.6, throughout. Final suspensions were in the same buffer at 1 mg protein/ml. Valinomycin was added (2 nmoles/mg protein) and the vesicles were then stored on ice until assayed.

All assays were performed at 25°C in 13×100 mm test tubes using an apparatus previously described[10] with O_2 used to aerate the samples. All radiolabelled substrates, electron donors, inhibitors, and antibiotics were added (0.5 ~1.0 μl) to 100 μl aliquots of the vesicles at the concentrations indicated using Hamilton syringes. Following removal from the ice, the vesicles were incubated for 3 minutes prior to addition of the electron donor and the radiolabelled substrate. Unless indicated otherwise, all antibiotics or inhibitors were added 1 minute prior to the addition of electron donors.

Uptake of either [$^3H_3$]-leu or $^{86}Rb^+$ was determined by rapid filtration on cellulose triacetate filters (0.45 μ) using 100 mM LiCl throughout to dilute and wash the samples. With the exception of the release studies, all time points represent the time between addition of the radiolabelled substrate and dilution with 100 mM LiCl. Filters were thoroughly dried and allowed to completely dissolve in scintillation fluid before counting. Each data point was corrected for nonspecific binding of radioactively labelled ligands using controls which were treated identically in the absence of an electron donor. Calculations of intravesicular concentrations of either [$^3H_3$]-leu or $^{86}Rb^+$ assumed a value of 2.2 μl internal volume/mg protein[12]. Membrane potentials determined from $^{86}Rb^+$ uptake experiments were calculated using the NERNST equation; $\Delta\psi = 58.8 \log [^{86}Rb^+]_{IN}/[^{86}Rb^+]_{OUT}$. It was assumed that the ratio of $[^{86}Rb^+]_{IN}/[^{86}Rb^+]_{OUT}$ was one in the absence of an electron donor ($\Delta\psi = 0$).

Protein was determined by the method of LOWRY[13].

Results

Influence of Octapeptin on Formation of the Membrane Potential

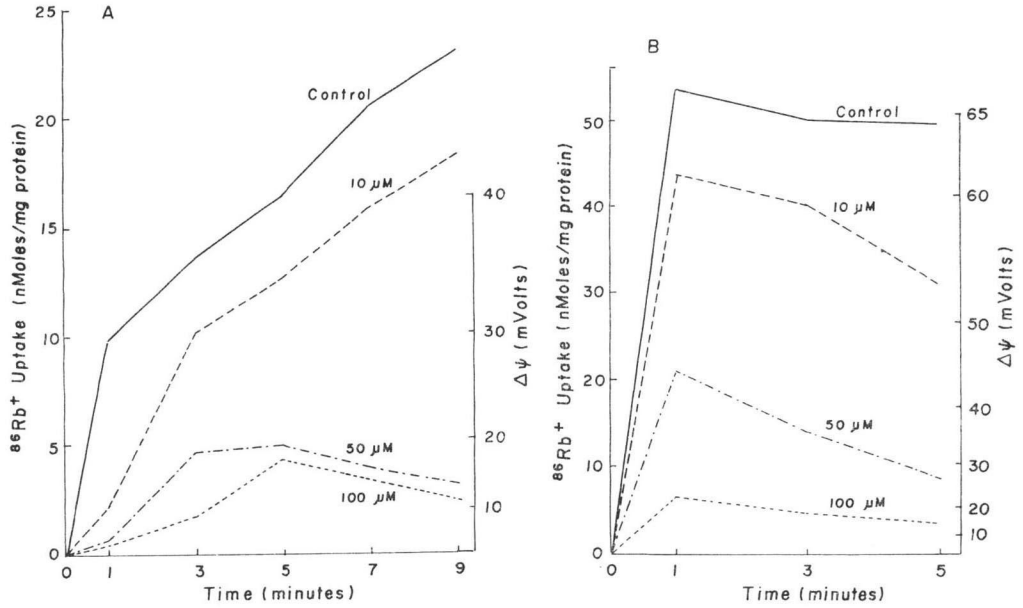
A number of radiolabelled ions have been used to monitor formation of the electrical potential generated by respiration in bacterial membrane vesicles[14~18]. These include the lipophilic cations dimethyl dibenzyl ammonium and triphenyl methyl phosphonium as well as $^{86}Rb^+$ (with valinomycin). Initial experiments indicated that octapeptin interacted quite strongly with the lipophilic cations, but not with $^{86}Rb^+$ and valinomycin. Therefore, the $^{86}Rb^+$-valinomycin transport system[18] was used to measure the formation of a membrane potential in the presence or absence of octapeptin.

The influence of octapeptin on the formation of the membrane potential by *E. coli* vesicles is presented in Fig. 1. Membrane potentials were established using either succinate (Fig. 1A) or Asc/PMS (Fig. 1B) as electron donors. In the absence of octapeptin, a membrane potential was rapidly generated using either respiratory substrate, although the rate and extent of membrane potential formation was lower with succinate compared to Asc/PMS. The maximum membrane potentials formed in the presence of succinate and Asc/PMS were 59 and 66 mV respectively. Formation of

Fig. 1. Succinate dependent (A) and Asc/PMS dependent (B) $^{86}Rb^+$ uptake by *E. coli* membrane vesicles in the presence of octapeptins concentrations of zero, 10 μM, 50 μM and 100 μM.

Membrane vesicles were prepared as described in Methods, at a final concentration of 1 mg membrane protein/ml.

Sodium succinate, sodium ascorbate, PMS and ^{86}RbCl were at concentrations of 20 mM, 20 mM, 100 μM and 2 mM, respectively.



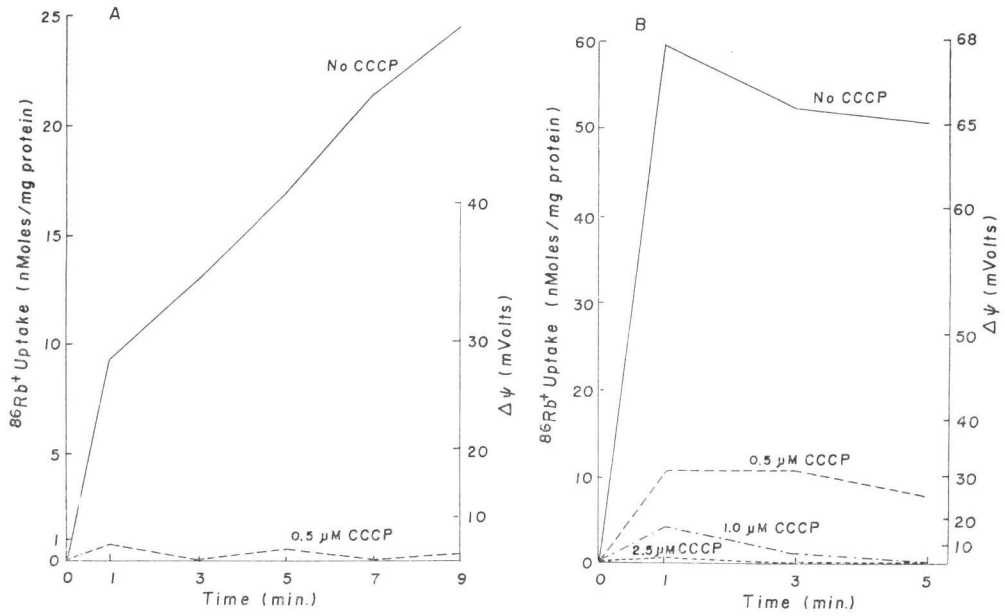
the membrane potential generated in the presence of either succinate or Asc/PMS was markedly inhibited by octapeptin (Fig. 1). For example, the maximum Asc/PMS generated membrane potentials were 66, 62, 44 and 25 mV in the presence of 0, 10, 50 and 100 μM octapeptin, respectively. Complete inhibition of membrane potential formation was achieved at 200 μM octapeptin (not shown).

The concentrations of octapeptin required to affect the membrane potential may seem relatively high since the minimum inhibitory concentration of the antibiotic for this *E. coli* strain is approximately two micromolar. However, relatively high concentrations of membrane vesicles were required for monitoring uptake of $^{86}Rb^+$ compared to concentrations of bacteria used in the determination of minimum inhibitory concentrations. These two measurements are most appropriately compared using the ratio of total octapeptin to membrane lipid required to affect bacterial growth or $^{86}Rb^+$ uptake. Formation of the membrane potential was markedly inhibited by 50 μM octapeptin which corresponds to a molar ratio of total octapeptin to membrane lipid phosphate of 0.50. The corresponding ratio at the minimum inhibitory concentration was 0.60 moles total octapeptin per mole of lipid phosphate. Thus, bacterial growth and membrane potential formation were inhibited at comparable ratios of antibiotic to membrane phospholipid. Binding studies using [^{3}H]-octapeptin and whole bacteria or isolated membranes indicated that there was approximately one mole of antibiotic bound per 50 moles of membrane phospholipid at minimum inhibitory concentrations of the peptide.

The effects of octapeptin on $^{86}Rb^+$ uptake were compared to CCCP since CCCP is a proton conductor which effectively collapses the electrical potential across bacterial membrane systems[17]. The influence of CCCP on membrane potentials generated in the presence of succinate or Asc/PMS

Fig. 2. Succinate dependent (A) and Asc/PMS dependent (B) $^{86}Rb^{+}$ uptake by *E. coli* membrane vesicles in the presence of CCCP.

Experimental conditions are identical to those indicated in Fig. 1.



was qualitatively similar to octapeptin (Fig. 2). CCCP inhibited both the rate and extent of membrane potential formation. However, CCCP was clearly more effective than octapeptin since comparable effects required 20~50-times higher octapeptin concentrations.

Influence of Octapeptin on Leucine Transport

The data discussed above illustrated that octapeptin inhibited the formation of the membrane potential in the presence of appropriate electron donors. Therefore, one would expect the antibiotic to inhibit active transport of metabolites which are driven by the membrane potential[14,17,19,20]. Fig. 3 illustrates that KABACK vesicles accumulated [^{3}H]-leucine against a concentration gradient in the presence of succinate (Fig. 3A) or Asc/PMS (Fig. 3B). Succinate driven accumulation of [^{3}H]-leucine was less effective than Asc/PMS.

Octapeptin inhibited leucine transport driven by either respiratory substrate (Fig. 3). Inhibition of leucine transport by octapeptin varied as a function of the peptide concentration. For example, Asc/PMS driven leucine transport was inhibited 25%, 66% and 100% at octapeptin concentrations of 10, 50 and 100 μM (Fig. 3B). By comparison, membrane potential formation was inhibited 38%, 82% and 94% at octapeptin concentrations of 10, 50 and 100 μM (Fig. 1B). A similar correlation was observed between octapeptin inhibition of membrane potential formation and leucine transport when succinate served as the electron donor (Fig. 1A and Fig. 3A). These observations strongly suggest that octapeptin inhibition of leucine transport is due to direct effects of the peptide on the membrane potential.

Inhibition of leucine transport by octapeptin could conceivably reflect either of two actions of the peptide; collapse of the electrochemical membrane gradient or enhancement of leucine membrane

permeability. The latter possibility was examined by preloading KABACK vesicles with [^{3}H]-leucine for 10 minutes in the presence of succinate and following the kinetics for release of leucine in the absence or presence of azide, azide and CCCP or azide and octapeptin (Fig. 4). It was not technically feasible to remove succinate and extravesicular [^{3}H]-leucine prior to examining back leakage of accumulated [^{3}H]-leucine. In the absence of any effectors, [^{3}H]-leucine levels continue to be maintained at about 0.20 nmoles/mg membrane protein. In the presence of azide, the net rate of leucine efflux was enhanced considerably indicating that continued electron transport was required to maintain leucine in the vesicles against a concentration gradient. Presumably, there is normally a high basal rate of leucine leakage counteracting leucine uptake in the presence of an electron donor. A combination of azide and CCCP, which blocks formation of the membrane potential and also relaxes the accumulated membrane potential, enhanced the net rate of leucine efflux even further. A similar rate of net leucine efflux was observed in the presence of azide and octapeptin. Thus, octapeptin did not appear to increase leucine permeability beyond that observed when generation of the membrane potential was blocked and the membrane potential was relaxed simultaneously.

Fig. 3. Succinate dependent (A) and Asc/PMS dependent (B) uptake of [^{3}H]-leucine by *E. coli* membrane vesicles in the presence or absence of octapeptin.

Membrane vesicles were at a concentration of 1 mg membrane protein/ml. Sodium succinate, sodium ascorbate, PMS and [^{3}H]-leucine were at concentrations of 20 mM, 20 mM, 100 μM and 500 μM, respectively. [^{3}H]-leucine uptake was monitored as described in Methods.

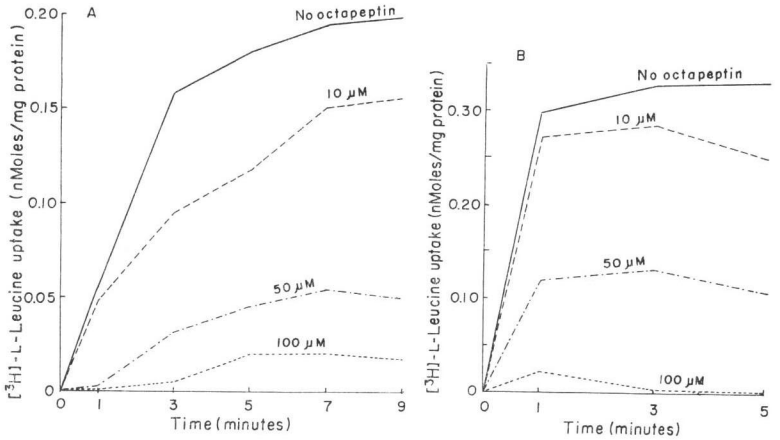
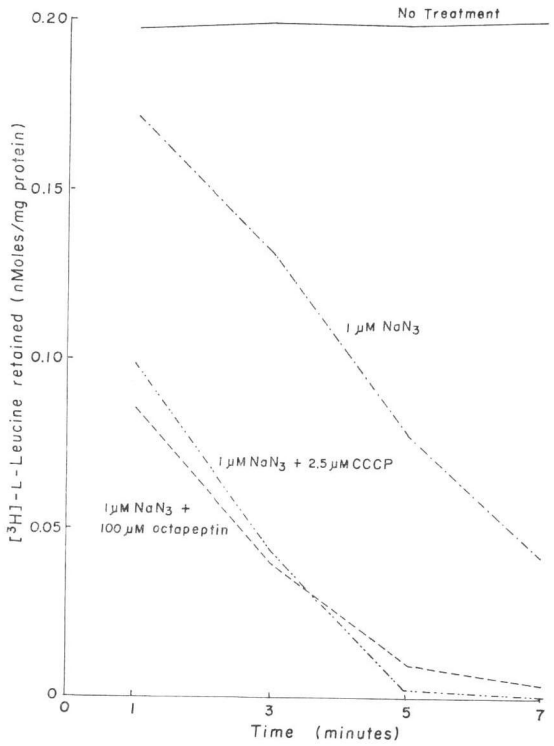


Fig. 4. Release of [^{3}H]-leucine from *E. coli* membrane vesicles preloaded for 10 minutes in the presence of succinate as described in Fig. 3, prior to treatment with effectors.

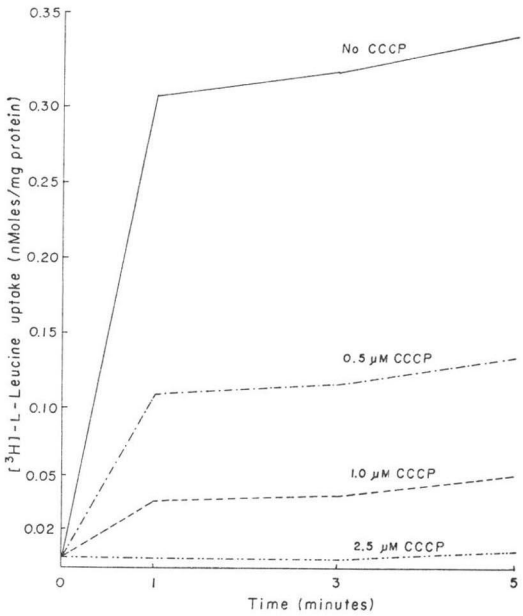


Inhibition of Leucine Transport by CCCP

The data discussed so far have shown that octapeptin is effective in inhibiting both the gene-

ration of a membrane potential and respiratory coupled transport of leucine. With Asc/PMS as a respiratory substrate, CCCP was effective in inhibiting formation of the membrane potential (Fig. 2) and is almost equally effective with respect to [^{3}H]-leucine transport (Fig. 5). In both cases, 2.5 μM CCCP was just sufficient for 100% inhibition, arguing for a direct relationship between the membrane potential and active transport of leucine.

Fig. 5. Asc/PMS dependent [^{3}H]-leucine uptake by *E. coli* membrane vesicles in the presence of CCCP.
Conditions identical to Fig. 2. Experimental conditions are identical to those indicated in Fig. 1.



Discussion

It has been previously demonstrated that octapeptin binds to bacterial membranes[6,11], causes observable changes in membrane structure[11] and increases membrane permeability with respect to protons and K^+[7]. These membrane permeability changes were restricted to small ions. Octapeptin treatment of bacterial cells did not result in release of larger molecular weight molecules such as nucleotides, amino acids or proteins[11]. On the basis of these observations, it was proposed that the primary action of octapeptin is to increase the membrane permeability for small ions and relax the membrane potential[6,7]. This proposal is supported by the data presented in this study. Octapeptin inhibited formation of the membrane potential generated in the presence of either succinate or Asc/PMS. The antibiotic also inhibited leucine transport which is clearly coupled to the bacterial membrane potential.

The molecular mechanism underlying octapeptin's effects on membrane permeability is not established. However, the peptide probably does not function as a proton ionophore since it bears a net positive charge of four at physiological pH and relatively high densities of bound antibiotic were required to inhibit bacterial growth and formation of the membrane potential. Furthermore, previous studies using octapeptin covalently attached to agarose beads illustrated that the antibiotic can affect the permeability and respiration of spheroplasts and protoplasts by interacting with the outside surface of vesicles[21]. It has been proposed that octapeptin interacts electrostatically with membrane lipid phosphates with insertion of the fatty acid tail into the center of the membrane. These interactions may cause disruption of membrane lipid packing and surface charge and lead to increased small ion permeability. This hypothetical model can account for all of the biochemical effects of octapeptin and is most consistent with the available data.

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