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# Chemical Modification of the Two Histidine and Single Cysteine Residues in the Channel-Forming Domain of Colicin E1

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**Summary:** The two histidine residues of COOH-terminal channel-forming peptides of colicin E1 were modified by addition of a carbethoxy group through pretreatment with diethylpyrocarbonate. The consequences of the modification were examined by the action of the altered product on both phospholipid vesicles and planar membranes. At pH 6, where activity is low, histidine modification resulted in a decrease of the single channel conductance from 20 pS to approximately 9 pS and a decrease in the selectivity for sodium relative to chloride, showing that histidine modification affected the permeability properties of the channel. At pH 4, where activity is high, the single channel conductance and ion selectivity were not significantly altered by histidine modification. The histidine modification assayed at pH 4 resulted in a threefold increase in the rate of $Cl^-$ efflux from asolectin vesicles, and a similar increase in conductance assayed with planar membranes. This conductance increase was inferred to arise from an increase in the fraction of bound histidine-modified colicin molecules forming channels at pH 4, since the increase in activity was not due to (i) an increase in binding of the modified peptide, (ii) a change in ion selectivity, (iii) a change of single channel conductance, or (iv) a change in the pH dependence of binding. The sole cysteine in the colicin molecule was modified in 6 M urea with 5,5'-dithiobis(2-nitrobenzoic acid). The activities of the colicin and its COOH-terminal tryptic peptide were found to be unaffected by cysteine modification, arguing against a role of (-SH) groups in protein insertion and/or channel formation.

**Key Words** colicin E1 · membrane channel · chemical modification

## Introduction

The colicin E1 molecule, a voltage-dependent ion channel has been shown to exert its lethal effect through its ionophoretic activity (reviewed by Luria & Suit, 1982; Cramer, Dankert & Uratani, 1983). Because the complete nucleotide and 522 amino acid sequence of the protein are known and the channel-forming domain is localized in the COOH-terminal region of the molecule, this is a well-defined system in which to study the molecular details of binding, insertion, and channel gating with model membranes. The role of particular amino acids in these processes can be studied by chemical modification or directed mutagenesis. The former approach has been taken in the present work to perturb residues, present in low stoichiometry, for which there is reason to believe an involvement in the mechanism of action. The possible involvement of the two histidine residues present at positions 427 and 440 in channel-forming activity had been suggested by the acidic pH dependence of in vitro activity of colicin E1 and its COOH-terminal tryptic peptide (Davidson, Cramer, Bishop & Brunden, 1984*b*). Specific modification of histidine with diethyl pyrocarbonate is known to inactivate enzyme function in several instances (Miles, 1977), presumably because of the presence of the bulky modifying group and/or the resulting decrease in the histidine pK. Insertion of diphtheria toxin (Wright, Marston & Goldstein, 1984) and epidermal growth hormone (Fong, Silver, Christman & Schwartz, 1960) into the membrane have been proposed to involve cysteine (-SH) transfer function. The COOH-terminus of the colicin contains a single cysteine residue at position 505 in the thirty-five residue nonpolar sequence that extends from residue 474 to 508. We therefore modified this single residue to determine if it affects either the formation or properties of the colicin channel.

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## Materials and Methods

### Preparation of Colicin E1 and its COOH-terminal Tryptic Peptide

Colicin E1 was purified from *Escherichia coli* strain JC411 (ColE1) grown on medium A with (0.05 g/liter) leucine, histidine, arginine, and methionine, and (0.01 g/liter) thiamine. Purification was according to Schwartz and Helinski (1971) as modified by Dankert et al. (1980). The COOH-terminal, $M_r$ 20,000 tryptic peptide fragment was prepared as described (Dankert et al., 1982) except that the Sephadex G-100 column was run at 4°C. The tryptic peptide was dialyzed against 3 × 1 liter of 0.5 M potassium phosphate buffer, pH 6.5, prior to ethoxyformylation with diethylpyrocarbonate (DEP).

### Ethoxyformylation (EF)

DEP[1] (Sigma) was freshly diluted with 100% ethanol prior to each experiment. The effective concentration of DEP was determined by reaction with 10 mM imidazole, pH 7.5 (Dickenson & Dickinson, 1975). The tryptic peptide in 0.5 M potassium phosphate buffer, pH 6.5, at concentrations of 0.1–0.5 mg/ml, was incubated at room temperature or 25°C with 0.05–0.15 mM DEP. The reaction was monitored spectrophotometrically between 320 and 230 nm, and the number of modified histidyl residues was calculated from the difference extinction coefficient of 3200 $M^{-1}$ $cm^{-1}$ at 238 nm between ethoxyformylated (EF-peptide) and untreated peptide (Miles, 1977).

The ethoxyformyl group was removed by incubation of the modified peptide with 0.33 M $NH_2OH \cdot HCl$, pH 7.5, at room temperature for 15–18 hr. The removal of the ethoxyformyl group from histidyl residues was monitored spectrophotometrically between 320 and 230 nm. Following $NH_2OH \cdot HCl$ incubation, the peptide was dialyzed against 3 × 1 liter of 0.5 M potassium phosphate buffer, pH 6.5, prior to assays of activity.

### Cysteine Modification

Colicin E1 or tryptic peptide in 0.1 M potassium phosphate, pH 7.0, was mixed with 8 M urea to yield a final urea concentration of 6.0 M. The urea-protein solution, with pH adjusted to 8.0 with NaOH, was incubated for 16 hr at 22°C with a 100-fold molar excess of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) (Sigma). The concentration of cysteine residues reacting was determined from the absorption at 412 nm of the thionitrobenzoate anion released stoichiometrically upon sulfhydryl group modification ($\varepsilon_m = 1.36 \times 10^4$ $M^{-1}$ $cm^{-1}$) (Means & Feeney, 1971). Unmodified controls were incubated with 6.0 M urea as described above. Prior to assays of activity, proteins were dialyzed against 3 × 1 liter of 0.1 M potassium phosphate buffer, pH 7.0.

### Assay of Cell Survival

Cells of the colicin E1-sensitive strain B/1,5 were stirred at 22°C for 5 min in the presence of 15–25 ng/ml of modified or unmodified colicin E1. Cell survival was determined by appropriate dilution and assay on agar plates.

### Preparation of Fused Asolectin Vesicles

Fused asolectin vesicles, mean diameter 0.5 μm, were prepared according to Davidson et al. (1984*b*). Asolectin (Associated Concentrales, Woodside, NY), purified by the method of Kagawa and Racker (1971), was suspended at 20 mg/ml in 10 mM dimethylglutaric acid (DMG), pH 4.0, containing 10 mM KCl and 90 mM NaCl. This suspension was sonicated for 15–30 min under $N_2$ in a Branson 12 bath type sonicator, frozen 5 min in an ethanol-dry ice bath, thawed at 22°C, and sonicated for 10–30 sec. Fusion was initiated by addition of 10 mM $Ca(NO_3)_2$, followed by a 30-min incubation at 22°C, and subsequent addition of EDTA (40 mM).

### Measurement of Chloride Efflux

Fused asolectin vesicles were diluted to a final lipid concentration of 0.2 mg/ml into a buffer containing 10 mM DMG, 10 mM calcium, 40 mM EDTA and $NO_3^-$ as the sole added anion. The concentrations of $KNO_3$ and $NaNO_3$ in the assay buffer were adjusted to produce variable $K^+$ gradients and, hence, diffusion potentials between +42 and −60 mV, inside to out in the presence of $5 \times 10^{-8}$ M valinomycin. $Cl^-$ efflux from vesicles in response to addition of tryptic peptide was monitored with a Radiometer (F1012Cl Chloride Selectrode) $Cl^-$-specific electrode and an amplifier of our own design. Rates of $Cl^-$ efflux were determined from the initial slopes of the $Cl^-$ efflux curve during the first 10 sec after colicin E1 or tryptic peptide addition. Any remaining trapped $Cl^-$ was released by the addition of Triton X-100 to a final concentration of 0.25%.

### Binding of Colicin Peptides to Membrane Vesicles

Fused asolectin vesicles were diluted into a buffer containing (in mM): 10, DMG; 1, $KNO_3$; 99, $NaNO_3$; 40, EDTA; 10, $Ca(NO_3)_2$, and NaOH for adjustment of pH. Tryptic peptide or EF-tryptic peptide labeled with [$^3$H]leucine (Davidson, Brunden & Cramer, 1985) was added to yield a final protein to lipid ratio of 1 : 100 (wt/wt). Following a 1-min incubation, duplicate samples were filtered through 0.2 μm membrane filters (Millipore, Type EG) which were presoaked and washed with a 2 mg/ml solution of bovine serum albumin. Filters were counted in a Searle 300 Scintillation counter to determine the amount of bound peptide trapped on the filter.

### Preparation of Planar Bilayer Membranes

Solvent-free planar membranes were prepared by the union of two monolayers (Montal & Mueller, 1972). Protein was added to the *cis* side. The voltages indicated were those of the *trans* compartment; the voltage of the *cis* side was set equal to 0 mV. The phospholipid used was asolectin Type IV-S (Sigma) which was washed in acetone (Kagawa & Racker, 1971). The planar membranes were bathed with symmetrical solutions of 1 M NaCl, 10 mM dimethylglutaric acid, 3 mM $CaCl_2$, 0.1 mM EDTA, titrated to the indicated pH with HCl or NaOH, unless otherwise stated. Salts and solvents were of reagent grade and used without fur-

[1] *Abbreviations:* EF, ethoxyformylated; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DEP, diethylpyrocarbonate, $\varepsilon_m$, molar extinction coefficient.

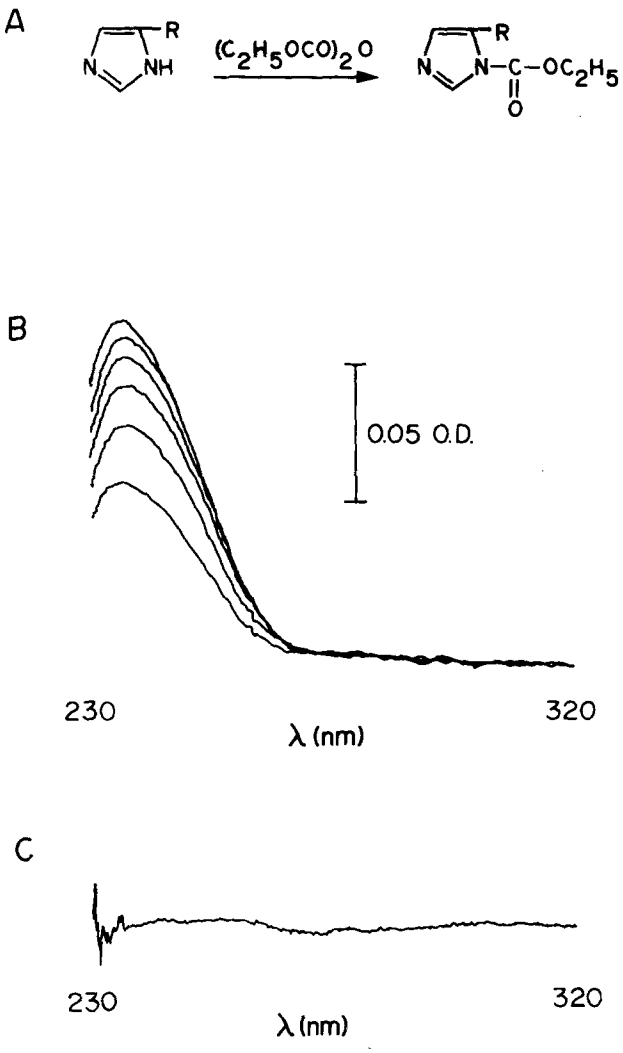


**Fig. 1.** (*A*) Ethoxyformylation reaction of imidazole. (*B*) A time course of ultraviolet absorbance difference spectrum (*A*) of colicin COOH-terminal tryptic peptide after DEP modification of histidyl residues, and (*C*) of the tryptic peptide after the DEP effect was reversed by removal of the carbethoxy group through incubation with 0.33 M $NH_2OH \cdot HCl$, pH 7.5, for 15 hr at 22°C

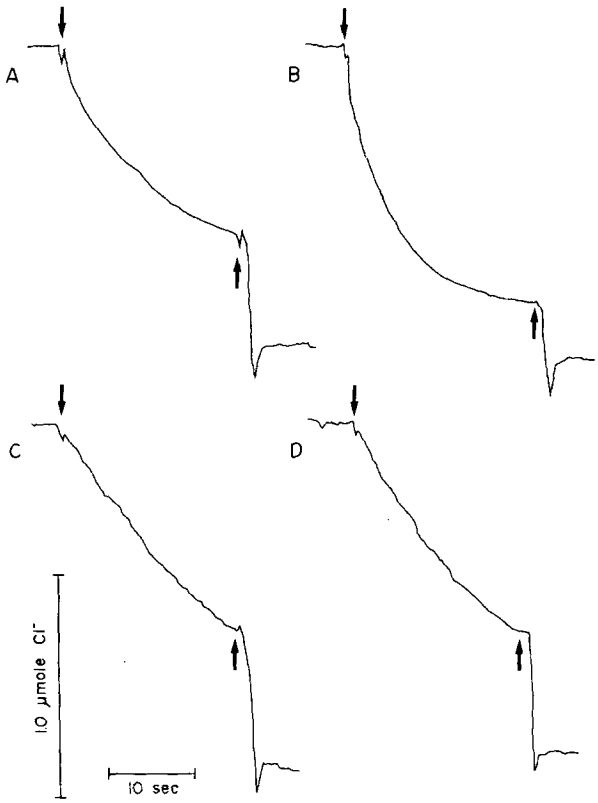


**Fig. 2.** $Cl^-$ efflux from fused asolectin vesicles in the presence of a $K^+$ diffusion potential (−60 mV) generated prior to addition of tryptic peptide. (*A*) tryptic peptide, 0.4 μg/ml; (*B*) EF-tryptic peptide, 0.4 μg/ml; (*C*) tryptic peptide, 0.4 μg/ml, incubated with 0.33 M $NH_2OH \cdot HCl$, pH 7.5, for 15 hr at 22°C, then dialyzed against 0.5 M KP buffer, pH 6.5; (*D*) EF-tryptic peptide, 0.4 μg/ml, from which the carbethoxy modifying group had been removed by $NH_2OH$ incubation as in *C*. The arrows (↓) and (↑) indicate, respectively, addition of peptide and Triton X-100 (0.25%)

ther purification. Millipore Milli-Q water (18 MΩ-cm) was used for all solutions.

## Results

### The Effect of Histidine Modification on the Activity of the Tryptic Peptide of Colicin E1

A time course of the ethoxyformylation reaction (Fig. 1*A*) of the colicin tryptic peptide by DEP is reflected in the difference spectrum between DEP-treated and untreated tryptic peptide (Fig. 1*B*). Employing $\varepsilon_m = 3200\ M^{-1}\ cm^{-1}$ as the extinction coefficient of the carbethoxy-histidyl residues (Miles, 1977), it can be calculated that stoichiometric modification of the two histidyl residues was achieved. This occurred without a decrease in absorption at 278 nm, characteristic of carbethoxy-tyrosyl residues (Burstein, Walsh & Neurath, 1974). The difference spectrum between (i) untreated peptide and (ii) DEP-modified peptide treated with hydroxylamine to remove the modifying group is shown in Fig. 1*C*. The lack of any difference in absorption at 238 nm indicates complete removal of the carbethoxy group from the histidyl residues (Miles, 1977).

The relative activity of the tryptic peptide and EF-peptide could be monitored by colicin peptide-induced $Cl^-$ efflux from asolectin vesicles. The rate of $Cl^-$ efflux in response to addition of the EF-peptide (Fig. 2*B*) was two- to threefold faster than the rate of $Cl^-$ efflux in response to the unmodified peptide (Fig. 2*A*). Following removal of the carbethoxy modifying group by $NH_2OH$, the $Cl^-$ efflux rate (Fig. 2*D*) was comparable to that caused by addi-

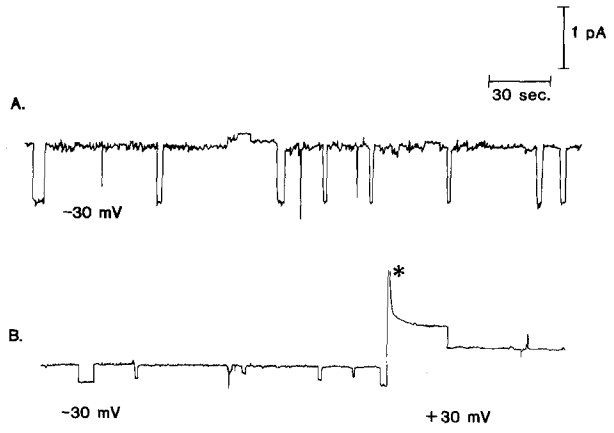


**Fig. 3.** Single channel records of unmodified (*A*) and EF-tryptic peptide (*B*). For the unmodified fragment (*A*) the membrane was voltage-clamped at −30 mV; For the EF-tryptic peptide (*B*), the voltage was switched from −30 to +30 mV at the asterisk

tion of unmodified peptide, indicating that the observed increase in peptide activity as assayed by $Cl^-$ efflux was due to the modification of histidyl residues. Incubation of unmodified peptide with $NH_2OH$ does not alter the activity of the peptide (Fig. 2*C*).

### Ion Selectivity and Single Channel Conductance

To determine if ion permeation through the conducting channel was altered by ethoxyformylation, ion selectivity and single channel properties were assayed. At pH 6 the unmodified and EF-tryptic peptide has reversal potentials, respectively, of 36.5 ± 1.6 mV ($n = 6$) (Bullock, Cohen, Dankert & Cramer, 1983) and 24.3 mV ± 1.6 ($n = 7$) (mean ± SD) in a 1 M *vs.* 0.1 M NaCl solution. The positive sign corresponds to the high salt side negative with respect to the low salt side, i.e., selectivity for $Na^+$ over $Cl^-$. After reversal of modification the measured reversal potential for the peptide was 33.2 mV ± 3.6 ($n = 6$). At pH 4 in a 1 M NaCl *vs.* 0.1 M NaCl solution, the unmodified fragment had a reversal potential of −37.2 mV ± 1 ($n = 6$) (high salt-side positive, i.e., selectivity for $Cl^-$ over $Na^+$), the EF-peptide had a reversal potential of −34.2 ± 1.6 mV ($n = 6$), and the EF-peptide after $NH_2OH$ incubation had a reversal potential of −37.8 mV ± 3.4 ($n = 8$). Thus, although histidine modification did cause an increase in $Cl^-$ selectivity at pH 6, the observed increase in activity at pH 4 assayed by $Cl^-$ efflux cannot be attributed to an increase in anion selectivity of the EF-peptide.

The single channel conductance at pH 6 of the EF-tryptic peptide (Fig. 3*B*) is smaller than the unmodified tryptic peptide (Fig. 3*A*). The single channel conductance of the tryptic peptide at pH 6 shows a broad distribution with a mean of approximately 20 pS (Fig. 4*A*, as in Davidson, Brunden, Cramer & Cohen, 1984*a*). The EF-tryptic peptide also exhibits a broad distribution for the single channel conductance, but the mean value decreased to 9 pS (Fig. 4*B*). At pH 4 in 1 M NaCl, the distributions of single channel conductances are again broad, with a mean value of 12 ± 5 pS (Fig. 4*C*) and 10 ± 4 pS (Fig. 4*D*) for the unmodified and EF-tryptic peptide, respectively. A population of larger channels (16–30 pS) is observed in the unmodified case which are not present with the EF-tryptic peptide, although the mean single channel conductance is not significantly altered. Thus, although the permeability properties of the channel were altered by the chemical modification at pH 6, there is no significant difference in either the single channel conductance or the channel selectivity at pH 4.

### pH Dependence of Activity and Binding of EF-Peptide

To investigate the possible role of the histidyl residues in the pH dependence of colicin ionophoretic activity in vitro (Davidson et al., 1984*b*) and to determine if the observed increase in activity of EF-peptide was dependent on pH, the $Cl^-$ efflux rates in response to the addition of unmodified and EF-peptide were assayed as a function of pH between 3.4 (the limit of vesicle stability) and 5.0 (Fig. 5). It is anticipated that the electron-withdrawing ability of the carbethoxy group will result in a substantial decrease, estimated to be about 2 units (Holbrook & Ingram, 1973), of the histidine pK. The concentrations of unmodified (0.27 μg/ml) and EF-peptide (0.1 μg/ml) were adjusted to give equivalent rates of $Cl^-$ efflux at pH 3.4, and the rates measured are expressed on a relative scale. Ethoxyformylation of histidine residues did not significantly affect the pH dependence of ionophoretic activity, and the increase in $Cl^-$ efflux rate in response to EF-peptide addition was observed at all pH values examined. The binding of [$^3$H]leucine-labeled EF-peptide to asolectin vesicles at external pH values between 3.6 and 6.0 was quantitated by incubating equivalent concentrations of [$^3$H] unmodified and EF-peptide with vesicles as described in Materials and Methods. The relative amount of bound peptide as a function of pH is shown in Fig. 6. The absolute amount of peptide bound at pH 3.6 was 35–40% of the total peptide present for both unmodified and EF-peptide. The similarity of the pH dependence of the activity of modified and unmodified peptide

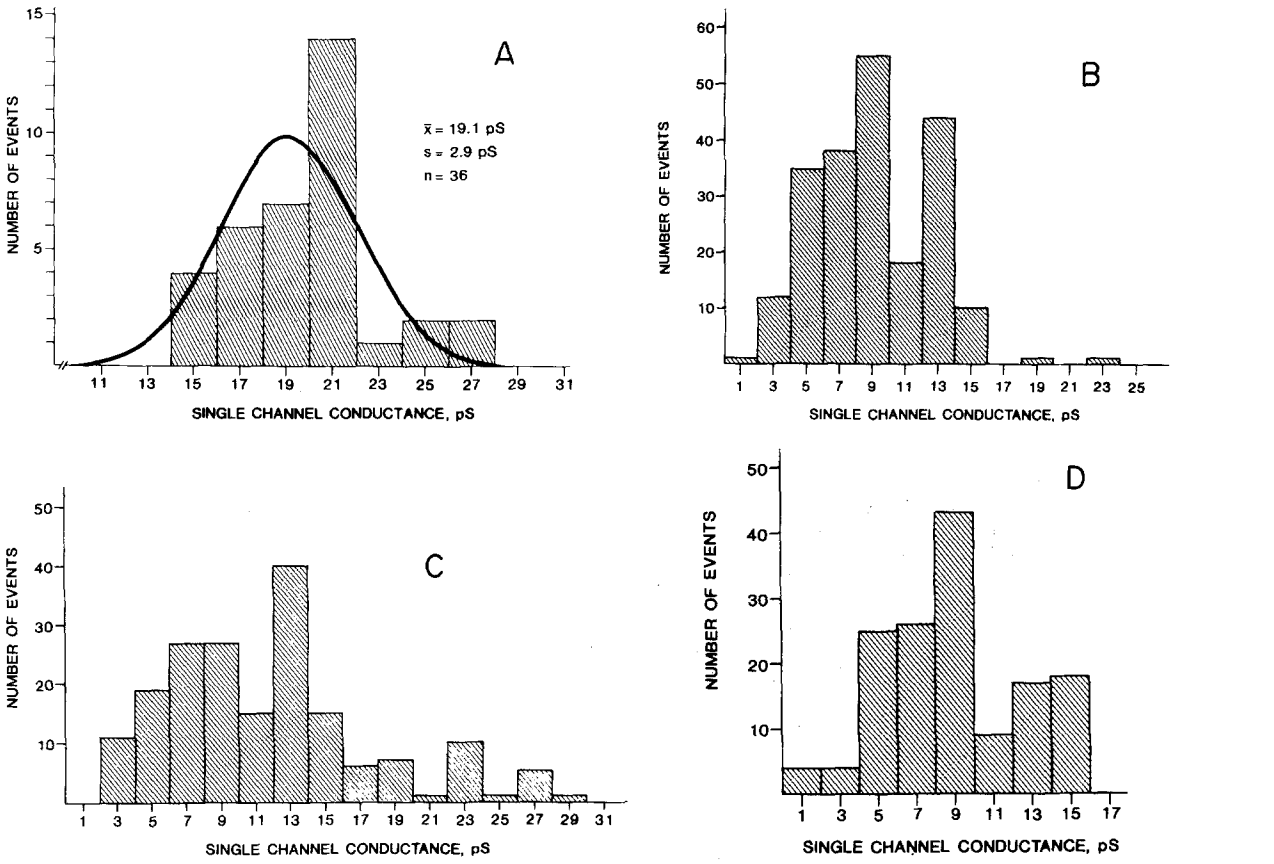


**Fig. 4.** Single channel conductance histograms of unmodified and EF-tryptic peptide. Protein was added to the *cis* side of an asolectin membrane bathed by symmetrical pH 6 (*A*,*B*) or pH 4 (*C*,*D*) solutions. (*A*) Tryptic peptide, pH 6.0. (*B*) EF-tryptic peptide, pH 6.0. (*C*) Tryptic peptide, pH 4.0. (*D*) EF-tryptic peptide, pH 4.0. For histogram (*A*), the single channel conductances were measured at −50 mV. For histograms *B*, *C*, and *D*, channels were pooled from membranes voltage clamped between −60 and 60 mV. For histogram *A* each event represents the opening of a different channel. The solid line is the Gaussian distribution normalized to the number of channels measured. The mean and standard deviation is indicated. For histograms *B*, *C*, and *D*, repetitive openings of the same channel *vs.* openings of different channels were not distinguished. Histogram *A* is reprinted with permission from Davidson et al. (1984*a*).

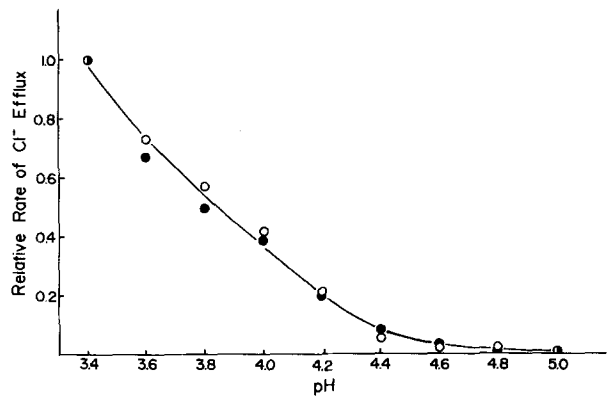


**Fig. 5.** pH dependence of $Cl^-$ efflux from asolectin vesicles caused by the addition of (●) EF or (○) unmodified tryptic peptide. The concentrations of EF- (0.1 μg/ml) and unmodified (0.27 μg/ml) peptide were adjusted to give equivalent rates of $Cl^-$ efflux at pH 3.4. A $K^+$-diffusion potential of −60 mV was generated prior to peptide addition

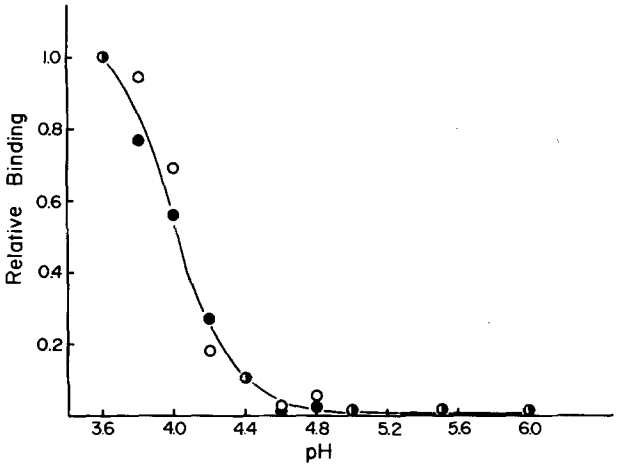


**Fig. 6.** The binding of EF- (●) and unmodified (○) [$^3$H]leucine-labeled peptide to fused asolectin vesicles as a function of pH. A −60 mV $K^+$-diffusion potential was imposed prior to peptide addition. Optimal binding corresponded to 36 and 37% of total peptide present for modified and unmodified peptide, respectively

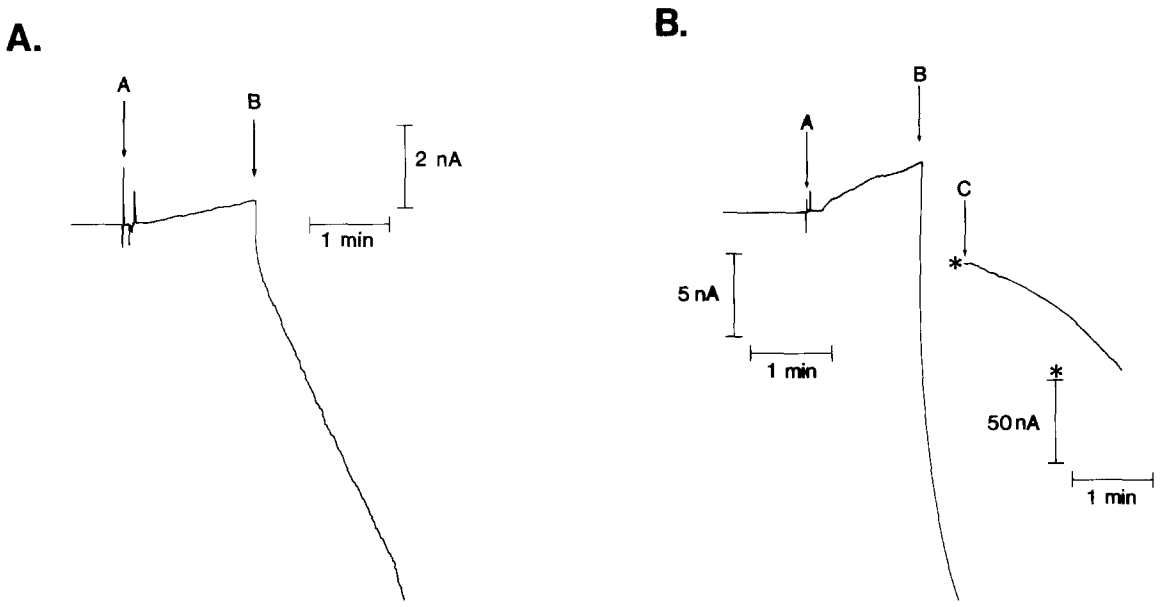


**Fig. 7.** Dependence of conductance of unmodified (*A*) and EF-peptide (*B*) on membrane potential in planar membranes. (*A*) Thermolytic peptide; (*B*) EF-tryptic peptide. Peptide was added (point *A*) to the *cis* side of a planar bilayer bathed in symmetrical pH 4 solutions and voltage-clamped at +20 mV. At point *B* the voltage was switched to −20 mV. (*B*) The gain of the chart recorder was reduced at point *C* to allow continuous monitoring of current. The asterisk denotes a change in scale

**Table.** Dependence of rate of change of current ($dI/dt$) of thermolytic peptide on ethoxyformylation[a]

| Peptide preparation | $dI/dt$ ($10^{-10}$ A/min) |
|---|---|
| I. Unmodified | 1 ± 1 ($n$ = 8) (mean ± SEM) |
| EF- | 30 ± 10 ($n$ = 10) |
| EF-, $NH_2OH$ | 3 ± 1 ($n$ = 3) |
| II. Unmodified | 6 ± 1 ($n$ = 8) |
| EF- | 33 ± 5 ($n$ = 12) |
| EF-, $NH_2OH$ | 12 ± 1 ($n$ = 6) |

[a] Conductance measured at −20 mV.

shows that protonation of the histidyl residues is not a factor in the acidic pH requirement for activity (Fig. 5) or binding (Fig. 6) of the tryptic peptide.

### Dependence on Membrane Potential of EF-Peptide Conductance Assayed in Planar Membranes

The dependence on membrane potential of tryptic (Bullock et al., 1983) and thermolytic peptide activity in planar membrane systems was similar (Fig. 7*A*) and much more pronounced than in membrane vesicles (Davidson et al., 1984*b*). EF-modification did not qualitatively affect the dependence of activity on membrane potential, as shown for the EF-tryptic peptide (Fig. 7*B*) and the thermolytic peptide (*data not shown*).

### Increase in Rate of Conductance Change after Ethoxyformylation

For two preparations of thermolytic peptide, the rate of current increase, $\frac{dI}{dt}$, in units of $10^{-10}$ A/min, was 1 ± 1 ($n$ = 8) (mean ± SEM) and 6 ± 1 ($n$ = 8) for unmodified peptide and 30 ± 10 ($n$ = 10) and 33 ± 5 ($n$ = 12) for EF-peptide (Table). Upon removal of the carbethoxy group by $NH_2OH$ incubation, the rate of conductance increase decreased to the unmodified level of 3 ± 1 ($n$ = 3) and 12 ± 1 ($n$ = 6) for both preparations. Thus, in agreement with the vesicle data (Fig. 2), the rate of current increase caused by the EF-peptide is greater than that in the presence of unmodified peptide, and the increase in rate of conductance is specific to histidyl modification.

### pH Dependence of Conductance of EF-Peptide

The rate of increase of conductance for EF-tryptic peptide increases as the pH of the *cis* compartment is lowered and is insensitive to changes in the *trans* pH. Decreasing the pH of the *cis* compartment from 6 to 5.5 results in an enhanced rate of conductance increase (Fig. 8). Lowering the pH of the *trans* compartment from 6 to 5.1 does not affect the activity. This phenomenon does not depend on pH gradients across the membrane and activity continues to in-

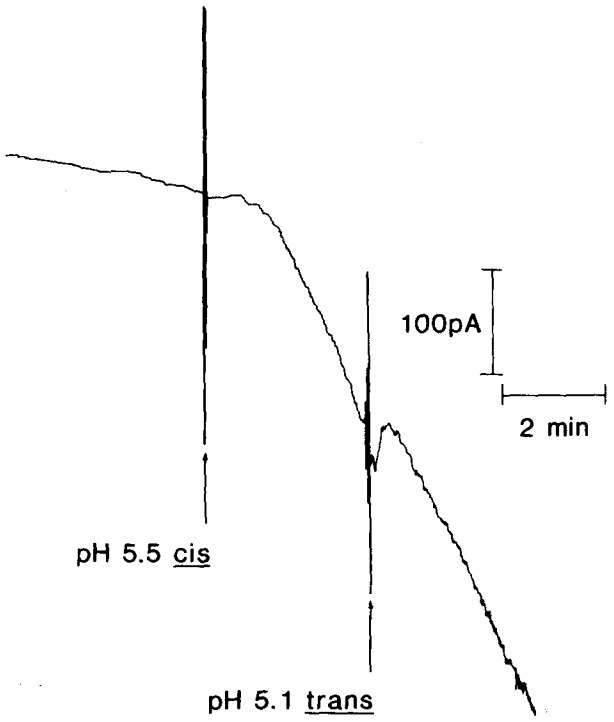


**Fig. 8.** Dependence of conductance of EF-peptide on pH. EF-tryptic peptide was added to the *cis* side of a planar membrane separating symmetrical pH 6 solutions. A transmembrane potential of −50 mV was initially applied to the membrane to obtain a steady increase in current. The pH of the *cis* side was first lowered to pH 5.5, with HCl, followed by the addition of HCl to the *trans* side to lower its pH to 5.1

crease as the pH of the *cis* side is lowered to 3.7. Similar results were obtained with unmodified tryptic peptide (Bullock et al., 1983). Thus, again in agreement with the results for vesicles (Figs. 5 and 6), ethoxyformylation of the histidyl residues is not critical for the observed increased activity at acidic pH.

### The Effect of Cysteine Modification on the Activity of Colicin E1 and Its COOH-Terminal Tryptic Peptide

$Cl^-$ efflux from fused asolectin vesicles in response to the addition of DTNB-modified (Fig. 9*A*) and unmodified (Fig. 9*B*) colicin E1, and DTNB-modified (Fig. 9*C*) and unmodified (Fig. 9*D*) tryptic peptide, are comparable. There is no significant effect of the thionitrobenzoate modifying group on the ionophoretic activity as assayed by $Cl^-$ efflux rates induced by addition of either the parental colicin E1 or the tryptic peptide, suggesting no involvement of Cys 505 in ionophoretic in vitro activity. DTNB modification could only be achieved in the presence of the denaturant, 6 M urea (*unpublished data*), which was removed by dialysis prior to assay of activity.

Bactericidal activity of DTNB-modified and unmodified colicin E1 was determined with the sensitive *E. coli* strain B/1,5. From the average of four modifications, the cysteine-modified colicin was found to be 80–90% as active as the unmodified colicin (*data not shown*), indicating no clear effect of the Cys 505 residue on colicin in vivo activity.

## Discussion

Domain analysis of proteolytic fragments of colicin E1 (Dankert et al., 1982; Bullock et al., 1983; Davidson et al., 1984*a*; Cleveland, Slatin, Finkelstein & Levinthal, 1983), along with sequence information (Yamada et al., 1982; Chan, Ohmori, Tomizawa & Lebowitz, 1985), has led to hypotheses concerning the role of particular amino acid residues that may be important in the structure of the colicin channel. Some residues are present at a small enough stoichiometry (1 or 2 residues/molecule) to allow meaningful conclusions to be drawn from specific chemical modification. Two histidyl residues, at position 427 and 440 in the colicin molecule, and the single cysteine at position 505 were chemically modified with DEP and DTNB, respectively, and the modified peptides were assayed for several parameters of activity.

The decrease in single channel conductance and increase in selectivity for chloride assayed at pH 6 after ethoxyformylation of His 427 and 440 suggests that these residues do affect channel properties. A preliminary model for the colicin channel (Davidson et al., 1984*b*) involved five membrane-spanning amphipathic α-helices, with His 427 and 440 toward one side of the bilayer on helices II and III (*not shown*). A likely effect of the bulky carbethoxy groups in the context of this model would be to narrow the channel lumen. This would explain the observed decrease in single channel conductance observed at pH 6. This conductance decrease may not be observable at pH 4 because the conductance is already limited at the latter pH in the absence of chemical modification. Similarly, the channel exhibits selectivity for anions over cations at acid pH (Raymond, Slatin & Finkelstein, 1985), thus perhaps masking any effect of the chemical modification at the single channel level on increased selectivity for anions at pH 4. The model does not, however, explain the threefold increase in $Cl^-$ efflux or current observed after modification. For this reason and its uncertain treatment of the two COOH-proximal membrane spanning peptides, the

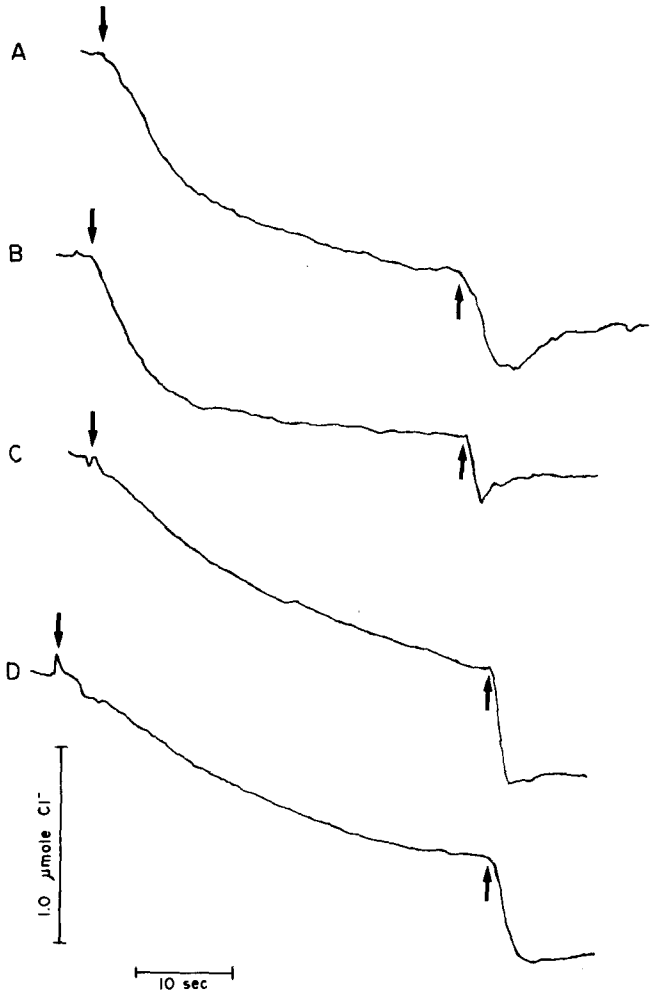


**Fig. 9.** $Cl^-$ efflux from asolectin vesicles caused by addition of DTNB-modified and unmodified colicin E1 and its tryptic peptide. Cysteine 505 was quantitatively modified by incubation with 100-fold molar excess of DTNB in 6.0 M urea. Samples were then dialyzed against 0.1 M potassium phosphate, pH 7. Unmodified controls were also incubated in 6.0 M urea and then dialyzed. The downward arrow indicates addition of 2 μg/ml of modified (*A*) and unmodified (*B*) colicin E1, and 1 μg/ml of modified (*C*) and unmodified (*D*) tryptic peptide. The upward arrow indicates addition of 0.25% Triton X-100 (final conc.) to release any remaining trapped $Cl^-$

model is considered to be at best partly correct. Its specific prediction regarding the role of Glu 468 in helix IV in the in vitro pH dependence of colicin activity is currently being tested (Shiver et al., 1986). Other models have also emphasized the role of α-helices in the colicin channel, but have argued that the COOH-terminal peptide cannot contain as many as five helices (Pattus et al., 1985). The three-helix dimer model of the latter authors also includes at least one histidine residue in a membrane-spanning peptide.

The activity of tryptic peptide modified at the two histidyl residues with DEP exhibited a threefold increase in activity assayed by peptide-induced $Cl^-$ efflux from $Cl^-$-loaded asolectin vesicles. The concentration of tryptic peptide could be increased ~threefold, or the concentration of EF-peptide similarly decreased, to achieve a comparable rate of $Cl^-$ efflux. Upon removal of the carbethoxy groups by incubation in the presence of $NH_2OH$, equivalent concentrations of peptide produced the same rate of $Cl^-$ efflux, providing further evidence that the observed alteration in activity is due to the specific modification of histidyl groups. This increase in activity is not due to an increase in the extent of binding of EF-peptide to the vesicle bilayer because binding of [$^3$H]leucine-labeled EF- and unmodified peptide was very similar, as was the pH dependence of binding (Fig. 6). Also, the increase in $Cl^-$ efflux is not due to greater $Cl^-$ efflux per channel because at pH 4 neither the single channel conductance nor the ion selectivity of the channel is significantly affected by DEP modification as determined from the planar bilayer experiments. We therefore conclude that the observed increase in activity of EF-peptide at pH 4 was due to an increased percentage of bound protein actually forming channels. This conclusion is supported by the greater magnitude of macroscopic current measured with planar membranes using EF-peptide as compared to unmodified peptide.

The dependence on pH of the activity of tryptic peptide has been titrated with the $Cl^-$ efflux assay system, and with the potential-indicating fluorescent probe Di-S-$C_3$-(5) (Davidson et al., 1984*b*). The effective pK for $Cl^-$ efflux due to peptide addition was ≤3.8. The most obvious candidate for the amino acid responsible for this pK would be acidic Glu or Asp residues. A histidine residue in a nucleophilic environment was initially considered as a candidate. This possibility is now excluded since there was no effect of modification of histidyl residues on the pH dependence of activity. In addition, the observed increase in EF-peptide activity relative to the unmodified control is independent of pH in the range 3.4–6.0.

The single Cys residue in the colicin E1 is at position 505, close to the COOH-terminal end of the 35 residue noncharged sequence, which extends from residue 474 to 508 (Cramer et al., 1983), and may act as an anchor for the channel in the membrane (Davidson et al., 1985). Colicin E1 and tryptic peptide modified at the single Cys-505 showed no alteration in in vitro or in vivo channel forming properties. The cysteine residues of diphtheria toxin, a toxin which must cross the plasma membrane to exert its lethal effect, appear critical for in vitro function (Wright et al., 1984). It was hypothe-

sized, similar to a mechanism for hormone internalization suggested by Fong et al. (1960), that sulfhydryl exchange reactions with membrane-bound sulfhydryl groups promote transport of a portion of the toxin through the membrane and serve as a covalent anchor to the membrane (Wright et al., 1984). The lack of any in vivo effect of DTNB modification of cysteinyl residues rules out such a mechanism in colicin E1 channel formation. The fact that the channel-forming colicin Ia, for which the sequence has recently been determined (J. Mankovich and J. Konisky, *manuscript submitted*), does not contain a cysteine, confirms the conclusion that the SH-exchange mechanism of toxin import into membranes is not applicable to the channel-forming colicins.

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