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Site-Directed Mutagenesis of Colicin E1 Provides Specific Attachment Sites for Spin Labels Whose Spectra Are Sensitive to Local Conformation

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ABSTRACT **Colicin E1 is an *E. coli* plasmid-encoded water-soluble protein that spontaneously inserts into lipid membranes to form a voltage-gated ion channel. We have employed a novel approach in which site-directed mutagenesis is used to provide highly specific attachment points for nitroxide spin labels. A series of colicin mutants, differing only by the position of a single cysteine residue, were prepared and selectively labeled at that cysteine. A hydrophilic sequence (398–406) within the C-terminal domain of the *water-soluble form* of the protein was investigated and exhibited an electron paramagnetic resonance (EPR) spectral periodicity strongly suggesting an amphiphilic α-helix. After removal of the N-terminus of the protein with trypsin, the spectra for this sequence indicate increased label mobility and a more flexible structure.**

Key words: α-helix, EPR, trypsinolysis

INTRODUCTION

Colicin E1 is a 56 kDa protein toxin secreted by *Escherichia coli* to kill other strains of *E. coli*. It binds to the vitamin B_{12} receptor on the outer membrane of target cells, translocates into the periplasmic space, and forms a nonselective ion channel in the inner membrane which uncouples oxidative phosphorylation[1] (for a review see reference 2). In vitro, this water-soluble protein spontaneously refolds and inserts into lipid membranes, in common with other bacterial toxins. Once inserted, it forms a voltage-gated channel.[3]

Studies of the function of proteolytic fragments of colicin E1 indicate that the intact molecule is organized into three functional domains corresponding to receptor binding, translocation, and channel activity (reviewed in reference 2). Trypsin digestion removes and degrades two-thirds of the molecule from the N-terminus, leaving a trypsin-resistant C-terminal domain of 186 amino acids (18 kDa) which retains the ability to bind to artificial phospholipid membranes and produce channel activity[4] as does a C-terminal peptide of 152 amino acids prepared by CNBr cleavage.[5] A short subfragment, though water insoluble, seems to retain channel activity.[6] Circular dichroism (CD) studies of the C-terminal tryptic peptide indicate a high percentage of α-helix in solution, which increases with the addition of octyl glucoside or upon binding to asolectin vesicles at low pH.[7] The crystal structure of the water-soluble form of the C-terminal peptide of a closely related protein, colicin A, has recently been determined.[8] This structure consists of a circumferential layer of amphiphilic helices surrounding a core of two hydrophobic helices, which are thought to be directly involved in membrane binding.

In order to study structure and function in this interesting protein, a new spin-labeling approach is being explored. The strategy is to provide specific attachment sites for the spin label in the protein using site-directed mutagenesis. In principle, a spin label can be placed at any desired point in the structure, and the EPR spectrum analyzed to provide information on the local environment. As will be shown in this communication, a set of sequential spin-labeled mutants can be used to provide more global information such as secondary structure. In the present work, attention is focused on the structure of the water-soluble form of the protein and its tryptic fragment.

A concern with the above approach is the possibility of structural perturbation upon amino acid substitution, particularly when the substitution involves a somewhat bulky group like the nitroxide spin label. The degree of single-site perturbation allowable for a given structure is an area of current research and a few general comments can be made based on recent results. First, substitutions of solvent-accessible surface groups have little effect on structure and function in the cases examined.[9,10] This is intuitively reasonable, and spin label side chains placed on external surfaces of proteins should

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provide nonperturbing probes. Several of the mutants considered below fall into this category. For side chains in the protein interior, it is important to preserve the polarity of the side chains and the total volume of the core in order to maintain a stable structure.[11] For water-soluble proteins, the core side chains are hydrophobic, while in membrane proteins they are of mixed polarity. The nitroxide side chain is relatively nonpolar, and is reasonably compatible with either situation. The volume of the spin label moiety added to cysteine is relatively small in the case of methanethiosulfonate spin label (~269 Å^3 compared to ~229 Å^3 for the tryptophan side chain), and may be accommodated in a protein core with some rearrangement of local side chains. The validity of detailed conclusions drawn from this approach will ultimately rest on demonstration of minimal perturbation provided by a variety of techniques such as CD, thermal denaturation, and urea denaturation.

In this initial study, two series of colicin mutants have been prepared within the C-terminal domain: at nine consecutive positions within a hydrophilic sequence (398–406) and at nine positions within a 35-residue hydrophobic sequence (482, 483, 485, 487–492). Mutants near the C-terminus (520) and the N-terminus of the C-terminal domain (340) were also prepared. Results from spin labeling studies of the water-soluble form of the protein suggest that the sequence 399–406 forms an amphiphilic α-helix, but refolds to a more flexible structure upon digestion with trypsin. The results of spin labeling 340 and 520 are also reported. Detailed spin labeling studies of the hydrophobic series will be reported in a later publication. A preliminary account of this work has appeared previously.[12] The results reported here deal only with the structure of the water-soluble form.

MATERIALS AND METHODS

Materials

Methanethiosulfonate spin label (MTSSL) was obtained from Reanal (Budapest, Hungary). Chromium oxalate (potassium trioxalatochromiate) was obtained from ICN Pharmaceuticals. Trypsin (chymotrypsin inhibited) was obtained from Worthington. *N*-Tosyl-*L*-lysine chloromethyl ketone (TLCK) was obtained from Boehringer Mannheim.

Synthesis of Pyridine Disulfide Spin Label

(2,2′-Dithiobis[5-{*N*-(4-(2,2,6,6-tetramethylpiperidine-1-oxyl))acetamido}pyridine]), referred to as pyridine disulfide spin label (PDSSL), was synthesized in a single step by the condensation of 4-amino-TEMPO and 6,6′-dithiodinicotinic acid in the presence of *i*-butylchloroformate.[13] Synthesis of 4-amino-TEMPO was according to Rozantsev[14] and was followed by distillation under high vacuum over BaO. 6,6′-Dithiodinicotinic acid (0.180 g, 0.00058 mol, Aldrich) was dissolved in 3 ml dry dimethylformamide (DMF). The reaction mixture was cooled to 0°C and 0.163 ml (0.00117 mol) triethylamine added, followed by 0.2 g (0.00146 mol) *i*-butylchloroformate (Kodak). After the reaction mixture was maintained at 0°C for 1 hour, 0.25 g (0.00146 mol) of 4-amino-TEMPO in 1 ml DMF was added with stirring and the mixture allowed to come to room temperature. After 2 hours the mixture was centrifuged and the precipitate discarded. DMF was removed from the sample and the sample redissolved in chloroform.

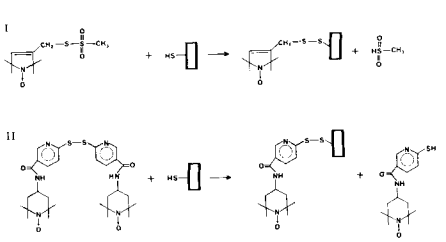
The sample was purified on a column (1.5 cm × 30 cm) packed with 15 g silica gel. The column was eluted first with pure chloroform and then with increasing methanol to 5%. The product eluted as the second orange band. Purity was checked by TLC on silica gel G plates (Analtech) with chloroform:methanol 19:1 as developing solvent. After spraying with H_2SO_4 followed by charring or spraying with fluorescein, the product was visible as a single spot with R_f = 0.35. The yield was 75%. The IR spectrum of the product (KBr pellet) exhibited an Amide I band at 1626 cm^{-1}, and the EPR spectrum showed a slow exchange interaction characteristic of a biradical. Double integration of the spectrum for a known weight concentration of the product gave results consistent with two spins per molecule (MW = 614). Stock solutions of PDSSL dissolved in acetonitrile were kept in a dark bottle and refrigerated. Fresh stock solutions were prepared when a precipitate began to form.

Preparation of Cysteine Mutants

A series of colicin E1 mutants, each with a cysteine or a methionine replacing the "wild-type" residue at a particular position and with the wild-type cysteine at position 505 replaced with glycine, were produced by site-directed mutagenesis.[6] The existence of a cysteine at the appropriate position in each mutant was verified by both gene sequencing and incorporation of [^{35}S]cysteine into the protein, while for the methionine mutants, CNBr was used to produce shorter fragments which were sized by gel electrophoresis.

Spin Labeling of Cysteine Mutants

The reaction of the two spin labels with sulfhydryl groups is outlined below.



In order to ensure the reduction of any protein disulfides that may have formed, solid dithiothreitol

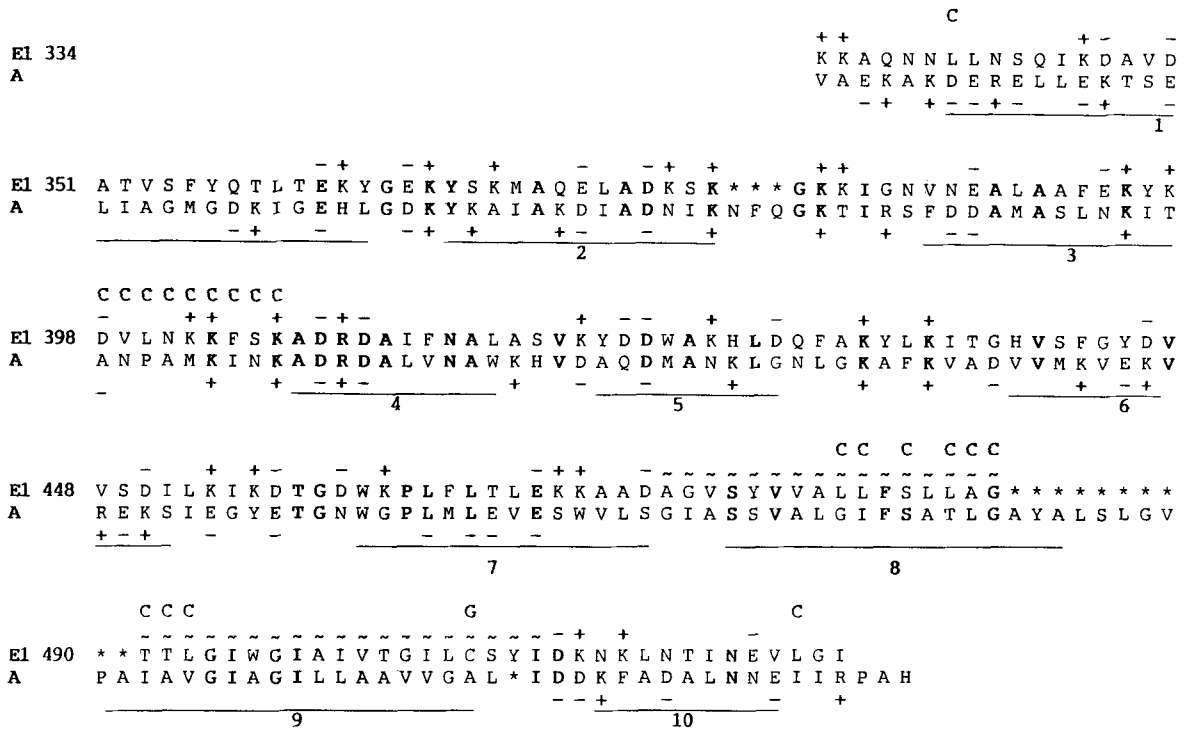


Fig. 1. Amino acid sequences for the C-terminal domain of colicins E1 and A with alignment by Parker et al.[5] Strictly conserved residues are in boldface. The extent of α-helices in the crystal structure of the C-terminal domain of colicin A are shown by horizontal lines.[8] The hydrophobic stretch in E1 is denoted by a wavy line. The position of cysteine mutants is indicated by a "C" above the E1 sequence and the replacement of the wild-type cysteine with glycine is indicated by a "G" above position 505.

(DTT) was added to 1 ml of the protein solution to make a final concentration of ~10 mM and allowed to stand for at least 1 hour at room temperature. DTT was then removed by passing the protein down a Sephadex G-25M column (PD-10, Pharmacia) equilibrated with buffer containing 150 mM NaCl, 10 mM MOPS, and 1 mM EDTA, pH 7. The concentration of protein in the collected fractions was assayed from the absorption at 278 nm ($\epsilon = 41{,}216\ M^{-1}\ cm^{-1}$). A 10-fold molar excess of spin label was then added to the reduced protein. For mutants 398 and 404, reaction with the spin label was accelerated by unfolding the molecule by the addition of solid urea to make a final concentration of 6 M. The reaction was allowed to proceed for at least 2 hours at room temperature in the dark. Unreacted spin and urea were removed on the column described above and equilibrated with the same buffer. The labeled protein was then concentrated with a Centricon 30 microconcentrator (Amicon). In most cases, stoichiometric labeling was verified by comparing the final protein concentration with the concentration of spin label, which was determined as described below. Spin labeled mutants were assayed for killing activity as previously described.[6]

Trypsin Digestion of Colicin

A stock solution of trypsin (10 μM) was prepared by dissolving the solid protease in the buffer described above and stored as frozen aliquots. Immediately before use, an aliquot was thawed and trypsin stock solution added to the colicin solution such that the final molar ratio of colicin to trypsin was ~50:1. Digestion was stopped after 2 or more hours by adding a large molar excess of TLCK.

EPR Measurements

EPR spectra were taken with a Varian E-109 X-band EPR spectrometer fitted with a loop gap resonator.[15] Samples of 5 μl were loaded in quartz capillaries sealed at one end. The magnetic field was stabilized with a Varian field/frequency lock accessory. Data acquisition and storage were controlled by a Nicolet 1280 computer interfaced to the spectrometer. Spin label concentrations for the labeled mutants were determined by double integration of EPR spectra followed by comparison with a standard aqueous 4-hydroxy-TEMPO solution.

RESULTS AND DISCUSSION

Site-Directed Cysteine Mutants Are Biologically Active

The C-terminal sequences of colicin E1, the closely related protein, colicin A, and the positions of cysteine mutants prepared in this work are shown in Figure 1. All colicin mutants with new Cys or Met residues inserted and the cyteine-less mutant

TABLE I. MTSSL- and PDSSL-Labeled Mutants

Mutant	Properties of MTSSL-labeled mutants			Properties of PDSSL-labeled mutants		
	Reactivity (-urea)	Label mobility	CrOx access	Reactivity (-urea)	Label mobility	CrOx access
340	+*	−	−	+	0	0
398†,‡	−	−,+	−,+	−	−,+	−,+
399†	+	+	+	+	+	+
400	0	−	0	−	−	0
401	+	+	+	+	+	+
402	+	+	+	+	+	+
403†	+	+	0	+	+	0
404‡	−	−	−	−	−,+	0,+
405	+	+	+	+	+	+
406	+	+	+	+	+	+
520†	+	−,+	−,+	+	0	0

*Very (+), moderately (0), not (−).
†Spectrum showing two or more clearly defined spin populations for MTSSL-labeled mutants. In cases where one component clearly dominates, only the motional state and accessibility of that component is noted.
‡Spectrum showing two clearly defined spin populations for PDSSL-labeled mutants.

(Cys^-) reported have about the same level of cell killing activity.[6] Only two mutant plasmids were not expressed (484 and 486), which may have been the result of endogenous proteolysis of misfolded nascent peptides within the cells. It has previously been reported that the mutants of the hydrophilic sequence (398–406) exhibit a trypsin cleavage pattern identical to wild type[6] and that nine other methionine mutants are biologically active.

EPR Spectra Are Sensitive to the Local Protein Environment

In this paper, we report the results of spin labeling of the colicin E1 cysteine mutants 340, 398–406, and 520. Spin label EPR spectra reflect nitroxide motional amplitude, frequency, and local solvent polarity. The reader is referred to two comprehensive monographs on spin labeling[16,17] for a discussion of the dependence of lineshape on these parameters. Analysis of these lineshapes by simulation can provide a detailed picture of the interactions with neighboring side chains in the protein.[18] In the present work we rely only on qualitative features of the spectra to derive information on the relative mobility and solvent accessibility of the nitroxide in the protein. It should be emphasized that the mobility of the nitroxide as estimated from the EPR spectra will primarily reflect motion of the nitroxide relative to the protein and not the rotational motion of the protein as a whole.

Two spin labels with complementary properties were employed in this work. PDSSL has the longer linking arm of the two and therefore exhibits more mobile, isotropic spectra. This has the effect of enhancing its sensitivity to protein conformational changes and to the presence of relaxation agents. On the other hand, its potential for hydrogen bonding with protein moieties, particularly in the hydrophobic membrane interior, may complicate the interpretation of spectra in terms of simple steric effects. MTSSL is smaller and therefore less likely to induce distortions in the native structure when labeling buried residues. Its shorter linking arm restricts the nitroxide to reporting on the environment closer to the protein backbone.

The Reactivity of Cysteine Mutants to Labeling With Nitroxides Varies With Position

Eight of the cysteine mutants prepared reacted in an approximately stoichiometric fashion with both spin labels over a time course of a few hours, suggesting accessibility of these sites to the aqueous phase. Complete labeling of mutant 400 with PDSSL was not achieved even after several days, suggesting very limited exposure. Mutants 398 and 404 reacted very slowly with both labels, indicating that these sites are buried in the native structure. These qualitative results are summarized in Table I. As will be shown below, the EPR spectra for this series of labeled mutants are consistent with these conclusions regarding accessibility. In preliminary work, we have also found that mutants in the hydrophobic sequence (482–492) react very slowly, suggesting that this sequence is also buried. The relative reactivity of site-directed cysteines has previously been used to draw similar inferences about protein structure.[19]

It has previously been shown that covalent modification of the cysteine in wild type (position 505) with a sulfhydryl reagent requires prior unfolding in urea and that the chemically modified and refolded protein is biologically active.[20] Unfolding in urea and refolding of the cysteine-less mutant, Cys^-, does not alter its trypsin cleavage pattern (see below). This indicates that colicin unfolding in urea is reversible and we have used this procedure to attach labels to 398 and 404.

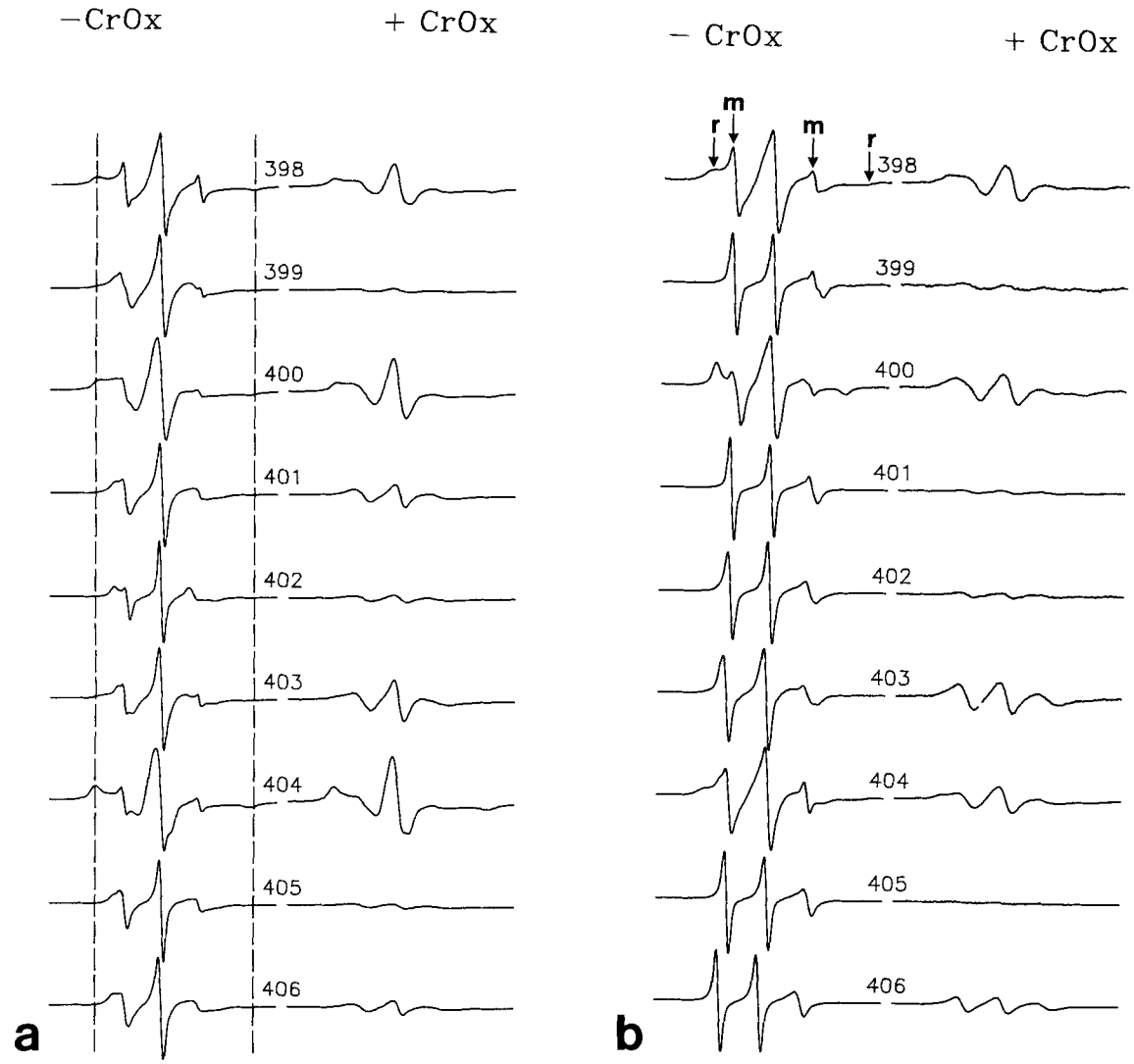


Fig. 2. (**a**) EPR spectra of MTSSL-labeled colicin mutants 398–406 in aqueous solution in the absence and presence of 50 mM CrOx. The vertical lines pass through the outermost hyperfine extrema of 404. The scan range is 100 G. (**b**) EPR spectra of PDSSL-labeled colicin mutants 398–406 in aqueous solution in the absence and presence of 50 mM CrOx. Arrows above 398 denote outermost hyperfine extrema of mobile (m) and motionally restricted (r) components. The scan range is 100 G.

Spin Label Mobility Varies With Position

The spin labeled mutant spectra in aqueous solution exhibit a variety of lineshapes depending upon the label and position along the peptide, as can be seen in Figures 2 and 3. Because of the small sample volume required by the loop-gap resonator (<5 μl), each spectrum required on the order of only 50 pmol of protein. The most useful qualitative information that can be readily extracted from such spectra is the relative mobility of the label. Consider first the spectra for the MTSSL-labeled mutants of the hydrophilic group in Figure 2a (left column). It can be seen that three of the spectra (398, 400, 404) are distinctive from their neighbors in the sequence by having widely spaced hyperfine extrema (see vertical lines), indicating that the label is strongly immobilized and buried in the protein interior. In contrast, the six other spectra have sharper spectral features and much more closely spaced hyperfine extrema. This indicates more rapid motion and suggests that these labels project from the protein surface in contact with water. On this qualitative basis, all of the spectra in Figure 2a may be classified as either mobile or motionally restricted. These results are summarized in the second column of Table I. A similar classification can be made of the spectra for PDSSL-labeled mutants of this sequence (Fig. 2b), which is summarized in the same table. The spectra

Fig. 3. EPR spectra of MTSSL- and PDSSL-labeled colicin mutants 340 and 520 in aqueous solution in the absence and presence of 50 mM CrOx. Position 340 is near the N-terminus of the C-terminal domain while position 520 is near the C-terminus. The scan range is 100 G.

for MTSSL- and PDSSL-labeled 340 and 520 are shown in Figure 3 and are also classified in Table I according to their mobility.

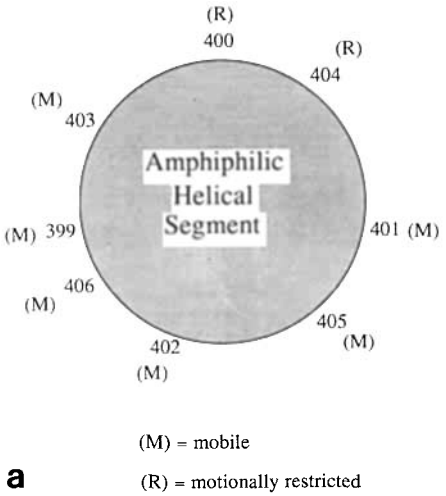
The spectra for the PDSSL-labeled mutants in Figure 2b reflect more rapid isotropic motion than the corresponding spectra for MTSSL-labeled mutants. A similar contrast is apparent for the two groups of spectra in Figure 3 (left column) as well. This is expected for a label with a longer linking arm between the nitroxide and the disulfide bond. The dynamics of the MTSSL label are more influenced by local side chain interactions and the low rotational frequency about the S–S bond. However, similar qualitative conclusions regarding the steric restrictions imposed by the site are obtained with the two labels. Each spectrum is unique and provides a characteristic signature for each label at each position. In future work employing spectral simulations, the spectral details will be an important source of information on local structure. However, for the present we simply note that the labels are qualitatively either mobile and unconstrained or motionally restricted.

Several of the spectra in Figures 2 and 3 have two prominent components, one moderately immobilized and the other mobile, and are identified accordingly in Table I. An example of this is 398-PDSSL in Figure 2b where a broad (motionally restricted) component and a sharp (mobile) component are identified with arrows. Such spectra suggest either that the labeled side chain is in slow equilibrium between two environments which impose different steric constraints or that the protein itself has two conformations. At present, we cannot distinguish between these possibilities. Because the amount of spin represented by a spectral line is proportional to the square of the linewidth, the sharp components usually comprise only a small fraction of the total spin. Several of the spectra of the MTSSL label (401, 402, 405, 406) appear to have multiple populations but are not indicated as such in the tables since anisotropic motions may account for the spectra.

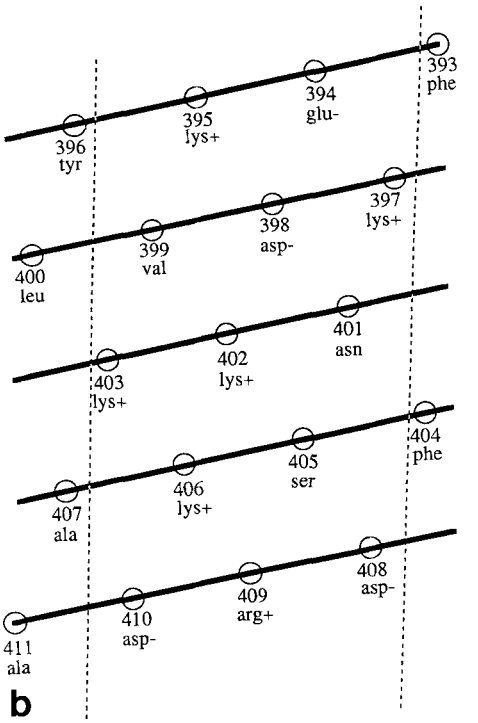
Spin Label Accessibility to Chromium Oxalate Varies With Position

The degree of accessibility of the labels to the aqueous phase is shown by the effect of addition of a paramagnetic reagent (Figs. 2 and 3, right column). Chromium oxalate (CrOx) affects nitroxide spectra by a Heisenberg exchange mechanism which requires a direct collision between the two species. The effect is a broadening of the spectral lines proportional to the direct collision frequency.[21] The nitroxides giving rise to sharp spectra corresponding to rapid motion are most affected. It can be seen that the sharp spectra generally become very broad with a correspondingly diminished amplitude, which is indicative of a reduced T_2 relaxation time due to Heisenberg exchange interaction. Thus, our inference that the mobile labels are exposed and in contact with water is demonstrated by their accessibility to CrOx. Within this group, the collision frequency clearly depends on position, presumably due to steric constraints imposed by neighboring side chains or local electrostatic fields (CrOx is anionic).

Immobilized labels are much less affected by the addition of CrOx, supporting the conclusion that these labels are at least partially buried in the protein. Labels showing composite spectra all have the mobile components strongly broadened, while the motionally restricted components are almost completely unaffected. This supports the interpretation that the mobile components arise from radical in contact with water, while the motionally restricted components are buried. The foregoing results are also summarized in Table I. It must be pointed out that the relatively long correlation time for the nitroxides in the more immobilized spectra make it possible that the line broadening contains contributions from dipolar as well as Heisenberg exchange mechanisms.[22] Thus, the slight broadening of the labels may not be due to direct collision, but to the close approach of CrOx. This method therefore may overestimate the accessibility of immobile components.



a



b

Fig. 4. (**a**) Helical wheel diagram of putative helical segment. "Mobile" and "motionally restricted" are qualitative references to the dynamics of a nitroxide group at the indicated positions. Note that the sites that give rise to motionally restricted spectra are located on the same face of the helix. (**b**) Helical net diagram for sequence 393–411. The dashed lines define the approximate limits of the highly charged polar face of the putative helix.

The Position Dependence of Label Properties Suggests Local Structure

Within the hydrophilic sequence 398–406, residues giving rise to motionally restricted and CrOx inaccessible spectra are located in similarly restrictive environments in the protein, but are not located adjacent to one another in the sequence as can be seen in Figure 2a and b and in Table I. They do, however, fall in the same *spatial* region if the sequence is mapped onto an α-helical geometry, as shown in Figure 4a. The immobile groups, 400 and 404, fall together on one face. Figure 4b shows that if the entire sequence from 393–411 was mapped onto an α-helix, it would be strongly amphiphilic and the motionally restricted groups would be located together on the nonpolar face. The polar surface is striking in its degree of polarity, with 10 of the 13 residues bearing a charge. For such a helix in a water-soluble protein, the nonpolar surface would be expected to face the interior of the protein. Hence the nitroxide groups on this surface would have hindered motion due to close packing with side chains on adjacent parts of the protein, as is in fact indicated by their EPR spectra. Thus the periodicities in reactivity, mobility, and accessibility for both nitroxide spin labels in this sequence are consistent with an α-helical structure. Note that 398, the first residue in the labeled sequence, does not fall into the periodic pattern apparent in the 399–406 region. Presumably, this could represent the termination of the helical region or tertiary interactions with nearby secondary structure. DeGrado and coworkers[23] employed similar reasoning in showing that the periodicity of the spectral properties of single tryptophan residues substituted into calmodulin-binding peptide was consistent with an α-helix.

pH Dependence of Conformation

It has been observed that the binding of colicin to asolectin liposomes increases with decreasing pH,[24] suggesting the possibility of a pH-dependent refolding of the protein in solution prior to binding. Refolding in solution with a change in pH has been observed for two other bacteriotoxins: *Pseudomonas* exotoxin A[25] and *Diphtheria* toxin.[26] Recently, Merrill and Cramer[27] have shown that a fluorescent label attached to the colicin wild-type cysteine at 505 is more exposed to water at pH 3.5 than at pH 6.0, indicating a partial unfolding of the molecule. Williams et al.[28] have reported rearrangements in the hydrophobic core of the protein between pH 5 and 6, as detected by proton exchange NMR. Refolding as a function of pH was not observed by circular dichroism.[7] However, circular dichroism is not sen-

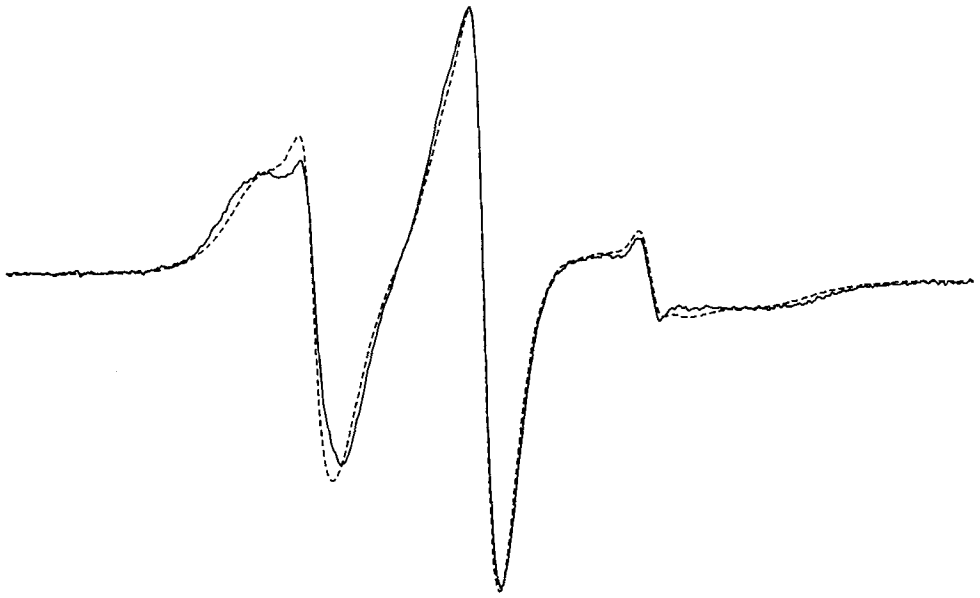


Fig. 5. Effect of pH on the spectrum of 340-PDSSL. Dashed line: pH 7. Solid line: pH 4. Spectra were normalized to the same amplitude. The scan range is 100 G.

sitive to changes in tertiary structure such as a rearrangement of domains.

We examined the spectra of many of the labeled mutants at pH 7 and 4. As can be seen in Figure 5, the spectrum for 340-PDSSL has more widely spaced outer extrema at pH 4, indicating more restricted mobility. This small but very definite change may indicate a rearrangement between protein domains with lowered pH, since 340 is very near to the tryptic cleavage site (position 336) which demarcates the C-terminal domain from the rest of the molecule. Several other labeled mutants in the amphiphilic sequence, including both the hydrophilic and hydrophobic faces of the putative helix, showed less significant spectral changes, possibly due to altered interactions with carboxyl side chains upon protonation (data not shown).

Comparison With the Crystal Structure of Colicin A

Recently, the crystal structure of a C-terminal thermolytic fragment of colicin A has been determined to 2.5 Å resolution.[8] Colicins E1 and A are very similar in terms of biological activity, and are expected to have homologous structures in the C-terminal domain (Fig. 1). It is therefore of interest to compare the above results with the structure of colicin A. The colicin A structure consists of 10 α-helices, 2 of which are entirely hydrophobic (8 and 9) and surrounded by 8 that are amphiphilic. Positions 340 and 520 of colicin E1 correspond to the beginning and end of amphiphilic helices 1 and 10, respectively, in the colicin A structure. These are both hydrophobic residues in the native molecule, and lie on the nonpolar faces of the helices. MTSSL spin labels at position 340 and 520 both show an immobile component, protected from CrOx, consistent with a structure for colicin E1 which resembles that for colicin A at these sites. Note, however, that PDSSL is much more mobile at 340 than MTSSL. This suggests that the site is close to a contact face between helices, and that the additional spacer arm length in PDSSL is sufficient to extend the label beyond local constraints.

The 398–406 sequence of colicin E1 is expected to correspond to the nonhelical loop region between helices 3 and 4 in the colicin A structure (Fig. 1). On the other hand, the evidence presented above suggests a helical conformation for this region in E1. There are three possible explanations for this difference. First, and most obvious, is that they are different molecules, and may simply have different structures in this region. This is supported to some extent by the fact that if the corresponding sequence in colicin A were mapped onto an α-helix, the resulting structure would not be particularly amphiphilic. Second, the position of the loop between helices 3 and 4 may be shifted. However, for the related colicins A, B, N, E1, Ia, and Ib, the sequences near the termini of helices 3 and 4 have a strictly conserved residue and the intervening loop contains a strictly conserved lysine at position 403 (Fig. 1). It therefore seems unlikely that the colicin A and E1 structures could be out of register in this region. Finally, the three-dimensional struc-

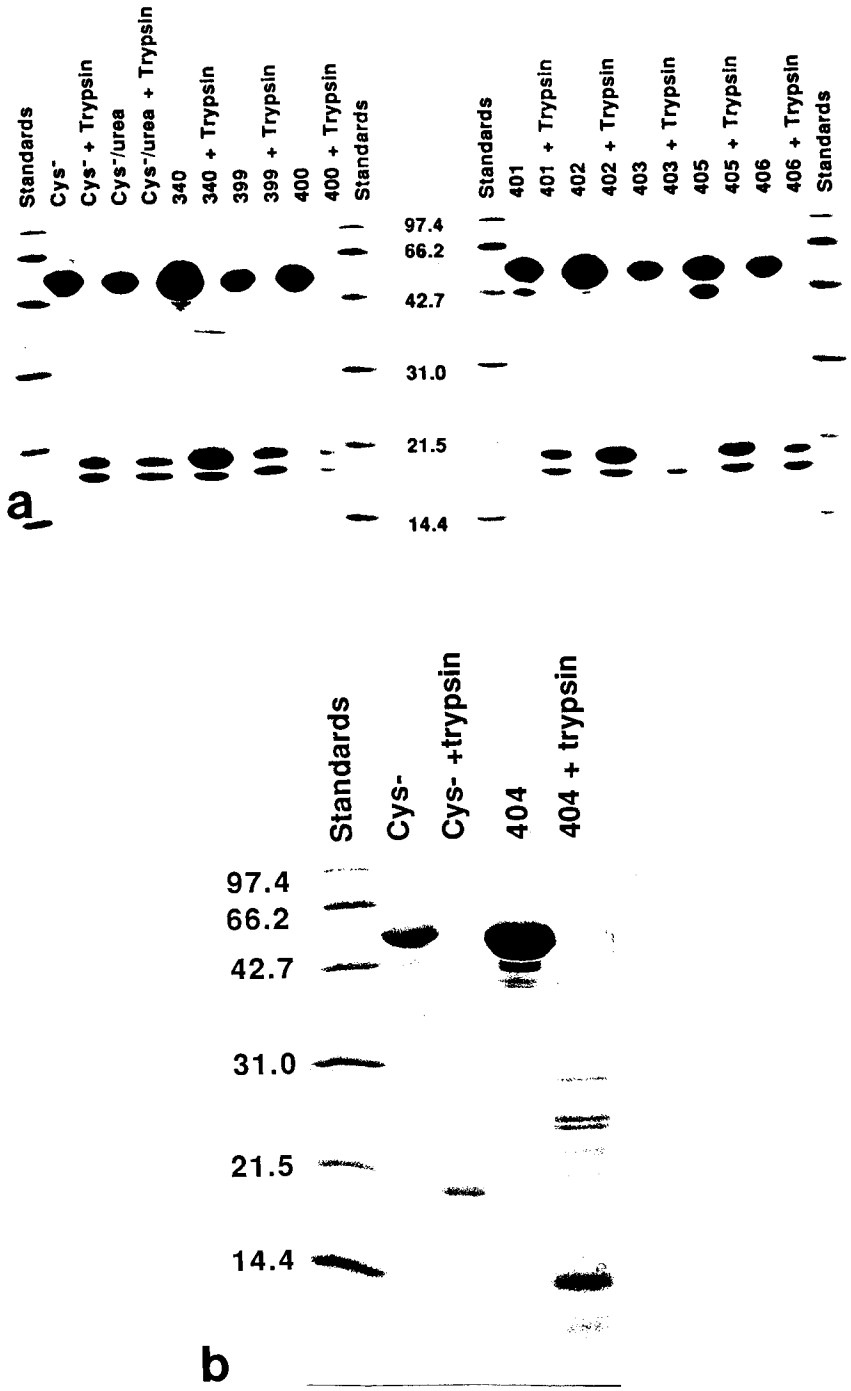


Fig. 6. (**a**) Trypsin digestion of MTSSL-labeled colicin mutants at positions 340, 399–403, and 405–406 as shown by SDS–PAGE. Also shown is trypsin digestion of mutant Cys⁻ and Cys⁻ treated with 6 M urea. Electrophoresis was carried out using a 15% acrylamide running gel and a 4% stacking gel, both containing 0.1% sodium dodecyl sulfate (SDS). Gels were stained with Coomassie blue. (**b**) Trypsin digestion of Cys⁻ and MTSSL-labeled 404. Digestion of 398 is not shown, but was very similar to 404.

tures for colicins E1 and A may in fact be similar, but the local conformation may be different for the whole molecule and for the C-terminal tryptic fragment. That this is the most likely explanation will be shown below.

Comparison of Tryptic Fragment With Whole Molecule

Mutant Cys⁻ (glycine substituted for cysteine at 505) and the MTSSL-labeled mutants 340 and 398–

406 were digested with trypsin and the cleavage pattern analyzed by SDS–PAGE (Fig. 6). It can be seen that for Cys^-, spin labeled mutant 340, and the labeled mutants on the polar surface of the putative helix (399, 401–403, 405, 406), the most important immediate cleavage products are a characteristic doublet of bands at about 18K. With further digestion, the upper band is converted to the lower band (data not shown), which is the relatively resistant C-terminal fragment. This pattern is indistinguishable from wild-type colicin E1.

The cleavage pattern is somewhat different for 400-MTSSL, which exhibits a light doublet of bands at 18 kDa, but with additional bands below 14K. This suggests that the presence of the MTSSL label at position 400 creates a sufficient distortion of the molecule in this region to accelerate proteolytic degradation by trypsin. This is perhaps not so surprising since there are three potential tryptic sites within three residues on either side of position 400. An altered cleavage pattern was also observed for MTSSL-labeled mutants 398 and 404, where the largest surviving band is at less than 14 kDa (Fig. 6b). Positions 398 and 404 are in fact potential tryptic sites. Since the Cys^- mutant shows a normal cleavage pattern after unfolding in 6 M urea followed by refolding, the altered cleavage pattern for these labeled mutants cannot be due solely to misfolding from treatment with urea. Evidently, the presence of the spin label on these hydrophobic surfaces causes local distortion of the conformation which exposes proteolytic cleavage sites. This may actually involve rather subtle changes in conformation. There are 24 lysines and 1 arginine in the C-terminal domain for a total of 25 potential trypsin cleavage sites. Conformational changes leading to increased exposure for any one of the sites may lead to rapid destruction of the peptide. The total intensity of the doublet of cleavage products of Cys^- decreases over time as observed by SDS–PAGE (data not shown) which suggests that the C-terminal domain is somewhat unstable even without the presence of a perturbing label. A similar loss of intensity over time can be seen in the trypsinolysis of wild type.[4] Therefore, even though the mutants labeled on hydrophobic contact surfaces must have some local deformations in structure, we do not have evidence to suggest that they are grossly altered in structure. All labeled mutants are able to bind to asolectin vesicles at pH 4 in a normal fashion and induce vesicle aggregation at high concentrations similar to the wild type. These findings will be reported in a subsequent publication.

As reported above, all of the MTSSL-labeled mutants from the polar surface of the putative helix and position 340 exhibited trypsin cleavage patterns essentially identical to Cys^-. At the same time, all of the PDSSL-labeled mutants that were tested (399–403, 405) exhibit biological activity indistinguishable from the unlabeled wild type. We conclude that these labeled mutants have a solution conformation very similar to both Cys^- and wild type. It is therefore reasonable to compare their EPR spectra before and after digestion with trypsin. The EPR spectra of trypsin-digested MTSSL-labeled mutants are shown in Figure 7. Position 340, only four residues from the trypsin cleavage site, becomes much more mobile with digestion as if it becomes part of a "frayed end" of the peptide. All labeled mutants on the hydrophilic surface of the putative helix also become much more mobile, indicating that the protein backbone has become more flexible. This apparently reflects conversion to a coil conformation. Thus, the structural information derived from EPR spin labeling of site-directed mutants of colicin E1 indicates that the N- and C-terminal domains are not structurally independent, and there are significant interactions which affect the structure in at least the 3–4 loop region. Significant interactions also exist between the receptor-binding domain of the N-terminus and the C-terminal domain of colicin A.[29] A possible concern in this study is that the more rapid rotational correlation time of the smaller tryp-

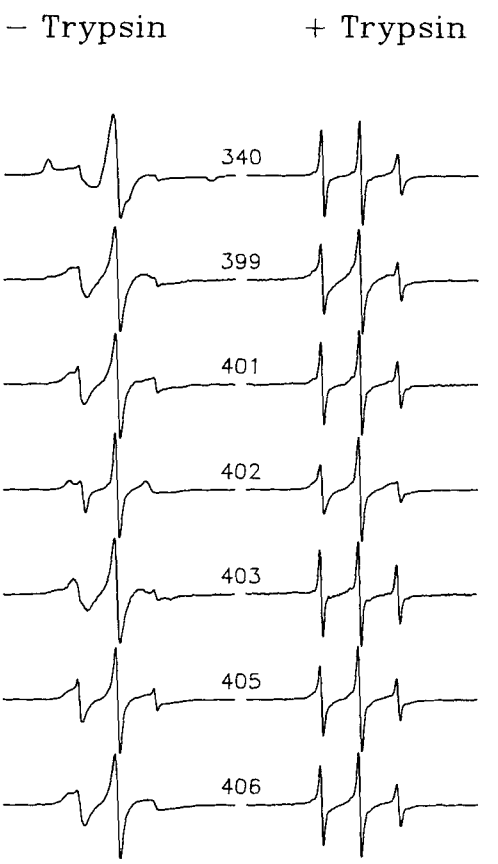


Fig. 7. The effect of tryptic digestion on the EPR spectra of MTSSL-labeled colicin mutants after 2 hours. The scan range is 100 G.

tic peptide may be responsible for the increased mobility of the nitroxide. However, the spectra indicate nitroxide τ_c on the order of 10^{-9} seconds while the calculated Stokes τ_c for a globular protein the size of the tryptic fragment is $\sim 2 \times 10^{-8}$ seconds, indicating little contribution from tumbling of the tryptic fragment. Future studies will reveal whether there are more differences in other regions of the structure. The evidence so far suggests that the tryptic fragment of colicin E1 in solution is similar in structure to that found for colicin A in the crystal, although the former and probably the latter are different in the intact molecules of the respective colicins.

The set of spin labeled mutants presented here, particularly those in the hydrophobic C-terminal domain, provides a promising tool for investigation of colicin interactions with membranes. Work is currently under way to extend this approach to areas beyond the current scope of crystallography such as the dynamics of insertion into membranes, the structure of the membrane bound protein, and the channel-gating mechanism. In particular, a few of the Met mutants were used to generate shortened fragments. Several of these fragments seemed to have properties inconsistent with a channel structure lined with α-helices.[6,30] For this reason, we are exploring by both EPR and electrophysiological measurements the possibility of a large, open β-structure as an alternative channel open-state.

CONCLUSIONS

All mutant proteins were biologically active as well as all spin labeled mutants that were tested (399–403, 405). There appeared to be little if any perturbation of the structure for labeled mutants with solvent-exposed spin labels, but labels that were buried in the structure did induce some perturbation, as detected by the pattern of trypsin cleavage.

The hydrophilic sequence 399–406 forms an amphiphilic α-helix in the water-soluble protein, but refolds to a more flexible conformation after removal of the N-terminus by trypsin digestion, illustrating the importance of tertiary interactions in stabilizing local secondary structure.

The novel method described here, combining genetic engineering with EPR spectroscopy, is a promising tool for the investigation of protein structure and dynamics.

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