Fourier transform infrared evidence for a predominantly alpha-helical structure of the membrane bound channel forming COOH-terminal peptide of colicin E1

P. Rath,* O. Bousché,* A. R. Merrill,‡ W. A. Cramer,‡ and K. J. Rothschild*

*Physics Department and Program in Cellular Biophysics, Boston University, Boston, Massachusetts 02215; and ‡Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907 USA

ABSTRACT   The structure of the membrane bound state of the 178-residue thermolytic COOH-terminal channel forming peptide of colicin E1 was studied by polarized Fourier transform infrared (FTIR) spectroscopy. This fragment was reconstituted into DMPC liposomes at varying peptide/lipid ratios ranging from 1/25–1/500. The amide I band frequency of the protein indicated a dominant α-helical secondary structure with limited β- and random structures. The amide I and II frequencies are at 1,656 and 1,546 cm^{-1}, close to the frequency of the amide I and II bands of rhodopsin, bacteriorhodopsin and other α-helical proteins. Polarized FTIR of oriented membranes revealed that the α-helices have an average orientation less than the magic angle, 54.6°, relative to the membrane normal. Almost all of the peptide groups in the membrane-bound channel protein undergo rapid hydrogen/deuterium (H/D) exchange. These results are contrasted to the α-helical membrane proteins, bacteriorhodopsin, and rhodopsin.

INTRODUCTION

Colicin E1 is one of a group of bactericidal proteins which exerts its lethal action in sensitive *E. coli* cells by forming an ion channel in the cytoplasmic membrane sufficiently conductive to depolarize the membrane and deenergize the cell (for a review see reference 1). Because it can be isolated in large quantity, genetically engineered, and exhibits voltage regulated channel gating, it is an ideal model for structure-function studies of membrane channel proteins. The channel forming function of colicin E1 can be localized in the COOH-terminal domain that is isolated as a protease-resistant peptide fragment (2) of 178 amino acid residues (3).

The group of channel-forming colicins includes Ia, Ib, B, N, A, and E1. The crystal structure of a soluble channel forming 204 residue carboxyl terminal peptide of colicin A has been solved to a resolution of 2.5 Å in aqueous solution (4). Well diffracting crystals have been obtained and the space group determined for thermolytic and tryptic channel peptides of colicin E1 (5). However, no crystallographic or physical information had yet been obtained on the structure of the colicin channel in the membrane.

Only one kind of membrane protein, the photosynthetic reaction center, has thus far been solved at high resolution by x-ray diffraction (6). No channel protein structure has been solved in this way. Thus, it is

necessary at present to gain an understanding of the membrane structure of the colicin channel protein by other approaches. Knowledge of the secondary structure would be important in itself and in the design of experiments to probe the identity of the *trans*-membrane peptide segments of the channel domain. The secondary structure of the water soluble forms of both colicin E1, assayed by either circular dichroism (7) or NMR spectroscopy (8), and colicin A (4) channel peptides is known to be predominantly α-helical. It is then natural to infer an α-helical structure in the membrane because it would involve a smaller number of steps in breaking and remaking H-bonds on conversion of the soluble to the membrane form. On the other hand, (*i*) it is clear that most of the α-helices identified in the structure of the soluble colicin A peptide are too short (average hydrophic helix length 13 residues compared to the average of 23–24 residues for bacteriorhodopsin [9] and 22 for the apolar segments of the helices of the photosynthetic reaction center [6, 10]) to span the bilayer; (*ii*) it has not been possible using predictive algorithms to identify the required number (presumably 4–6 in a monomeric channel) of membrane spanning hydrophobic and amphipathic helices (11, 12) required for a fully functional channel. The possibility of a β-barrel type structure was discussed (12) because of (*a*) the existence of several 9–11 residue segments of relatively high amphipathicity whose position coincided with local peaks of hydrophobicity in the colicin E1 channel domain (12), and (*b*) the precedent of the β-barrel structure of the *E. coli* OmpF porin protein (13).

The technique of Fourier transform infrared (FTIR)

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¹*Abbreviations used in this paper:* a.u., absorbance units; bR, bacteriorhodopsin; DMPC, dimyristoyl phosphatidylcholine; FTIR, Fourier transform infrared; H/D, hydrogen/deuterium.

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spectroscopy can be used to probe the secondary structure of proteins in membranes (14, 15). Using this technique, the *E. coli* porin protein *in situ* has been determined to consist of predominantly β structure (16), whereas in contrast bacteriorhodopsin, rhodopsin, cytochrome oxidase, and Ca-ATPase were found to be predominantly α-helical (17, 18, 19, 20, 21, 22). The utilization of several FTIR related methods including polarized FTIR, resolution enhancement techniques, and hydrogen/deuterium (H/D) exchange can provide additional information about secondary structure in a membrane-bound protein (14, 22, 23). In the present study, the channel-forming COOH-terminal thermolytic peptide of colicin E1 was incorporated into DMPC liposomes at varying peptide/lipid ratios and the secondary structure of the incorporated peptide was determined.

MATERIALS AND METHODS

Preparation of colicin E1 and its carboxyl-terminal peptide

The 178-residue channel peptide was prepared by treatment of intact colicin E1 with thermolysin as previously described (24). DMPC-channel peptide proteoliposomes were prepared according to the procedure of Uratani and Cramer (25) using lipids from Avanti Polar Lipids, Inc. (Birmingham, AL). The peptide/lipid ratio was varied from ~20–500. All the preparations were made in 10 mM Hepes buffer at pH 7 with 100 mM KCl.

FTIR measurements

Films of colicin E1 channel peptide suitable for FTIR measurements were prepared by slowly air-drying ~0.4 mg of colicin E1 peptide reconstituted at various lipid/protein ratios (24:1, 57:1, and 440:1) on AgCl windows. Peptide films were rehumidified by exposure to water saturated air for ~5 min and then sealed in a specially constructed IR cell using a second AgCl window. For the deuteration studies, a drop of D₂O (99.8%, Sigma Chemical Co., St. Louis, MO) was added to a dry colicin E1 peptide film on a AgCl window inside a dry box, and excess D₂O was removed by shaking. The film was then immediately inserted into the IR cell.

The room temperature unpolarized FTIR measurements were carried out at 2 cm⁻¹ resolution either in a model 740 spectrometer equipped with TGS detector or 60SX spectrometer equipped with a MCT-B detector (Nicolet Analytical Corporation, Madison, WI). Polarization measurements were made on both dry and humidified films at 77 K with a KRS-5 wire grid polarizer using procedures previously described (26). Interferograms were apodized using a modified Happ-Genzel window. Fourier deconvolution technique was used to obtain resolution enhancement of the amide I and II bands as described (23, 27, 28).

Analysis of dichroism was based on the method of Rothschild and Clark (17). The index of refraction of the film was taken to be 1.6 which is between the values of the refractive index of an air-dried DMPC film (1.55) (29) and the index typically found for polypeptides in IR (1.7) (30). Dichroic ratios at 0, 15, 30, and 45 degrees were measured in order to calculate the average helix tilt angle.

Protein assay of the sample on AgCl window

The amount of peptide superficially bound to liposomes on the AgCl window was determined by repeated washing (12 times) of the window with 10 mM Hepes buffer, pH 7, containing 0.1 M KCl to remove the lipids from the support. The liposome suspension was sedimented by centrifuging at 100,000 g for 30 min and the peptide content in the supernatant was determined by its absorbance at 280 nm. < 10% of the peptide was found to be superficially bound to the liposomes.

RESULTS

FTIR spectra of the colicin E1 thermolytic channel peptide reconstituted at lipid/protein ratios of 24:1, 57:1, and 440:1, recorded at room temperature, are presented in Fig. 1. The frequencies of the amide I (ν_I, peptide carbonyl stretch) and amide II (ν_{II}, N–H bend) bands are near 1,656 and 1,546 cm⁻¹, respectively, which is characteristic of α-helical secondary structure (15). Amide I and II bands are observed at similar frequencies for other predominantly α-helical membrane proteins including rhodopsin in photoreceptor membrane and reconstituted membranes (ν_I = 1,655 cm⁻¹ and ν_{II} = 1,545 cm⁻¹) (18, 31) and bacteriorhodopsin (ν_I = 1,659 cm⁻¹ and
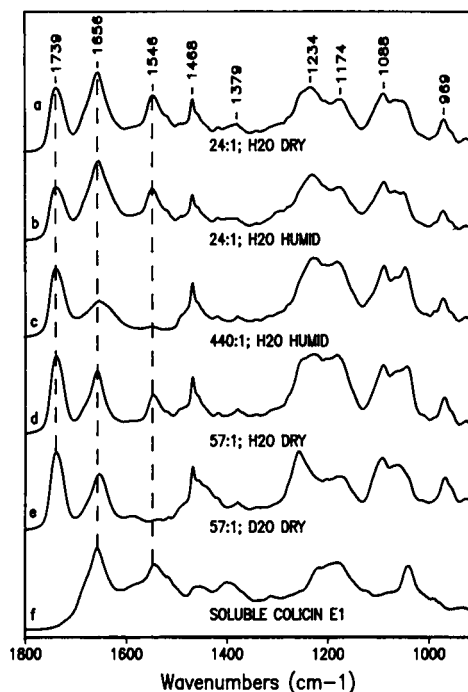

FIGURE 1 FTIR spectra of colicin E1 channel forming peptide reconstituted into DMPC lipids at various lipid/protein ratios and formed into multilamellar film (*a–f*). Spectrum (*f*) is derived from the soluble colicin E1 peptide. All the preparations were made in 10 mM Hepes and 100 mM KCl.

ν_{II} = 1,545 cm^{-1}) (17, 22, 32). The amide A band of the colicin E1 channel peptide appears at 3,297 cm^{-1} in contrast to 3,310 cm^{-1} for bR and 3,295 cm^{-1} for rhodopsin (22, 31). The higher frequency of the amide I and A bands in bacteriorhodopsin relative to colicin E1 and rhodopsin may be due to the two-dimensional packing of bR in the purple membrane (see Discussion).

The amide band frequencies of the channel peptide were found to be relatively insensitive to both changes in hydration level and lipid/protein ratio over a wide-range (Fig. 1, a–e). Even at a lipid/protein ratio of 500 and much higher water content than shown for the sample in Fig. 1, the amide I and II bands were still close to 1,656 and 1,545 cm^{-1} (unpublished data). Furthermore the intensity of these bands decreases relative to that of the lipid band (i.e., 1,739 cm^{-1}) as the lipid/protein ratio increases. This indicates that the assignment of these bands is correct and that the α-helical structure detected in the colicin E1 channel forming peptide is not induced by dehydration or by an abnormally low lipid/protein ratio. Thus, we conclude that the structure of the membrane-bound form of the colicin E1 channel forming peptide fragment is predominantly α-helical. The FTIR spectrum of the soluble thermolytic fragment of colicin E1 is also presented in Fig. 1 f. The amide I and amide II bands for this soluble peptide appear at frequencies (1,657 and 1,545 cm^{-1}) very similar to those observed for the bound form, confirming that the soluble form of colicin E1 is also predominantly α-helical under the conditions of these experiments. However, several of the bands associated with lipid vibrations at 1,739, 1,234, 1,088, and 969 cm^{-1} (31) are not present in the soluble peptide as expected.

To probe the accessibility of the membrane-bound colicin E1 channel forming peptide fragment to the aqueous medium, we studied the effects of exposing a dried film to D$_2$O. As seen in Fig. 2, after only 5 min a decrease of ~80% occurs in the ratio of the amide I/amide II bands which reflects the percentage of amide peptide groups which undergo hydrogen/deuterium exchange (22). After more than 72 h this ratio has decreased by ~90%. A concomitant rise in intensity occurs near 1,450 cm^{-1}, which corresponds to the amide II' band of the deuterated peptide group (predominantly ND bending mode) (15). The band due to the tyrosine vibration near 1,515 cm^{-1}, which shifts to 1,513 cm^{-1} in D$_2$O (33), is clearly observed because of the reduction in intensity of the amide II band. A frequency shift is also observed in the amide I band from 1,656 to 1,654 cm^{-1}, as expected for α-helical structure but not random structure (34). Thus, it appears that a significant fraction of the colicin E1 channel secondary structure undergoes very rapid H/D exchange.
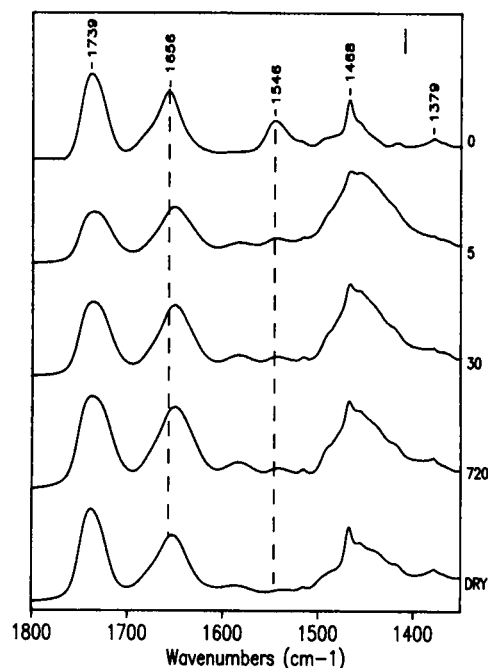
Additional information about the secondary structure

FIGURE 2 FTIR spectra of colicin E1 channel forming peptide fragment reconstituted into DMPC lipids (lipid/protein = 57:1) at different times (right side of figure) after exposure to D$_2$O. The vertical bar represents an absorbance of 0.1 a.u. The increase in absorbance at 1,450 cm^{-1} band in the 5 min spectrum is due to amide-II' band of the deuterated peptide groups and partially from HOD. The reduction in intensity at this frequency is due to the gradual loss of D$_2$O from 5–720 min. The bottom spectrum (dry) is from the same sample used for the 0–720 min H/D exchange.

of proteins can be obtained by studying the resolution enhanced amide I and II band profiles of spectra (27, 35) recorded at low temperature (22, 28). This method enables individual subcomponents of the amide I and II bands to be detected which correspond to different types of secondary structure (35). As seen in Fig. 3, the Fourier-deconvolved spectrum of colicin E1 channel forming peptide has several subcomponent bands in the amide I and II region in addition to the main amide I and II bands at 1,656 and 1,553 cm^{-1}, respectively (note that the amide II band shifts from 1,546 to 1,553 cm^{-1} upon cooling to 77 K). All of these bands were also detected using second derivative resolution enhancement (data not shown). Significantly, almost all of these bands appear at a similar, although in some cases slightly higher, frequency in bR (see also reference 36). Differences in intensity observed for the strong bands at 1,641 and 1,530 cm^{-1} in bR, arise in part from the C = N and C = C stretch of the retinylidene chromophore of bR (22, 28). The shift of the main amide I band of bR to a higher frequency than is typical of α-helical proteins has been attributed to helix–helix
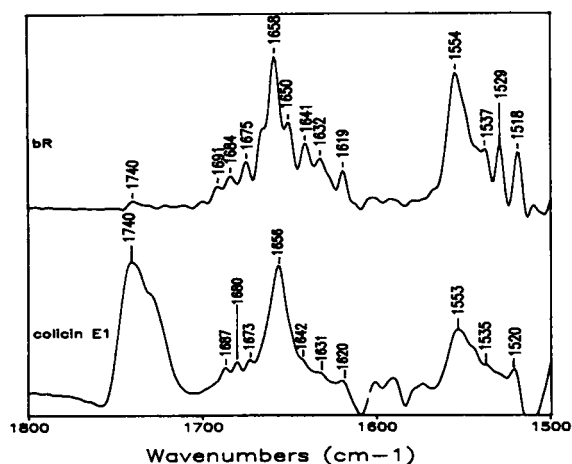
FIGURE 3 Resolution enhancement of the spectrum from a dry film of colicin E1 peptide (lipid/protein = 57:1) and bacteriorhodopsin at 77 K. Spectra were Fourier self-deconvolved using commercially available software LabCalc(TM) (Galactic Industries Corp.). The parameters used were gamma = 3.5 and filter = 0.35.
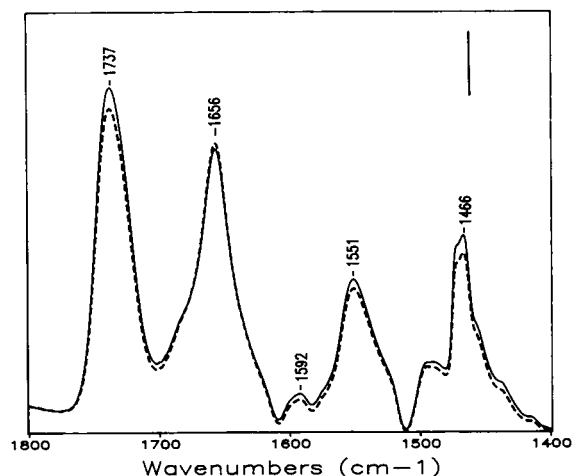
FIGURE 4 Polarized FTIR of dry film of colicin E1 peptide (lipid: protein = 57:1) at a tilt angle of 45° for perpendicular and parallel polarization recorded at 77 K. The solid line curve is for incident light polarized perpendicular to the plane of incidence (parallel to the sample plane) and the dashed curve is for light polarized parallel to the plane of incidence. The vertical bar represents an absorbance of 0.05 a.u.

coupling (37, 38), or alternatively to an α_{II} conformation (39). The bands > 1,656 cm^{-1} are characteristic of β-type structure including β-turns. In the amide II region, in addition to the major band at 1,553 cm^{-1}, a small band is found at 1,520 cm^{-1} which may be due to a tyrosine mode (33). It should also be noted that purple membrane lacks lipids with ester carbonyls, thus explaining the absence of intense bands near 1,740 cm^{-1}. In summary, the resolution enhancement reveals that the vibrational spectra of bR and the colicin E1 channel forming peptide in the amide I and II regions are very similar. These spectra are consistent with a predominantly α-helical structure for the channel peptide with possible low levels of β-structure.

Polarized FTIR can be used to probe for net orientation of protein structural components including the α-helices in oriented membranes. In the case of bacteriorhodopsin as well as several other predominantly α-helical membrane proteins including rhodopsin (18, 40), cytochrome oxidase (21) and the photosynthetic reaction center (19, 41, 42), the transmembrane orientation of these helices causes a characteristic dichroism in the amide I and II bands when the sample normal is tilted relative to the incident light direction. The amide I band increases in intensity for light polarized parallel to the incident plane whereas the amide II band decreases in intensity (17), provided the net order parameter for the helix tilt corresponds to an angle <54.6° (the magic angle relative to the sample normal). The same dichroism behavior is found both for dry (Fig. 4) and hydrated films of colicin E1 (data not shown). This indicates that the net orientation of the α-helices in the colicin E1

channel forming peptide in reconstituted membranes is <54.6 degrees from the sample normal. A more exact estimate would require that the orientation (i.e., the mosaic spread) of the reconstituted membranes relative to the orienting substrate be known (see below).

DISCUSSION

The structural changes accompanying the transition of colicin E1 and its active channel peptide from the water soluble to the membrane-bound state are likely to be extensive but are not understood in any detail. It is well established that the active COOH-terminal channel peptides of colicin E1 and A are α-helical in solution. The secondary structure of the channel peptide in the membrane had not yet been determined experimentally, although it has been considered by predictive methods (11, 12, 43). Such an analysis is made difficult by (a) the largely amphipathic nature of the channel, and the questions of (b) its minimum length, and (c) whether it is a monomer or a dimer.

The present study establishes that the structure of the colicin E1 channel forming peptide consists predominantly of α-helical structure in both the soluble and membrane bound forms. For example, the frequency of the amide I, II, and A peptide bands are similar to those in bacteriorhodopsin, which contains predominantly α-helical structure (9, 17, 22) and almost identical to

bovine rhodopsin (14, 31). The main difference in the spectral comparison with bR is the higher frequency of the amide I and A bands, which may arise from increased helix–helix interactions due to the two-dimensional crystal packing of bR (37, 38). Just as in the case of bR, there are at least three subcomponent bands in the amide I region of colicin E1 $>1,670$ cm^{-1} and three $<1,650$ cm^{-1} at similar frequencies which are detected by resolution enhancement. The "standard" assignment for these bands is to β-type structure such as β-turns (35). However, similar bands are also observed in other highly α-helical proteins such as alamethicin (44). An interesting possibility is that these bands arise in part from less intense normal modes of the α-helix which are expected for finite length chains (45). Alternatively, some of these bands might arise from vibrational modes of amino acid side chains.

It is possible that the conformation we detect for colicin E1 channel forming peptide reconstituted into DMPC membrane vesicles is not the same as the native conformation of the channel in the physiologically active form of colicin E1. For example, recent evidence indicates that the amount of peptide inserted exogenously into the liposomes is increased by $\sim 50\%$ in the presence of a membrane potential. The labeling experiments of Merrill and Cramer (46) indicated that ~ 70–80 residues composing two α-helical hairpin helices of the channel peptide are inserted into the bilayer in the absence of a membrane potential and another 35–40 residues are inserted in the presence of the potential. Of the remaining noninserted residues of the 178-amino acid channel peptide, it is thought that a large portion are organized into surface-seeking α-helices oriented parallel to the membrane plane. Because no such potential is present under the conditions of our FTIR measurements, it is possible that we are not measuring the physiologically active form of colicin E1 which depolarizes the membrane. However, in this study the colicin peptide used was not added exogenously to preformed liposomes, but was reconstituted with lipids to form proteoliposomes which under these conditions exhibit active channel function (25).

The orientation of the α-helical domains of the membrane bound form of colicin E1 remains undetermined. Our polarized FTIR measurements demonstrate that the average α-helix orientation is $<54.6°$, the magic angle relative to the membrane normal. This could reflect an average of two to three segments with orientations which are different from one another. The segment oriented perpendicular to the membrane would be the one to two transmembrane helical hairpins incorporated into the bilayer in the absence of a membrane potential (46) whose orientation is near 0°, and a second amphipathic extramembrane segment (i.e., the segment span-

ning the Ala-425 → Trp-460 region) which is parallel to the membrane surface (orientation 90°) in the absence of the potential. The polarization data for the 178-residue channel peptide are rather accurately accounted for by (a) ~ 70 residues in two helical hairpins oriented perpendicular to the membrane plane; (b) ~ 35 residues oriented parallel to the membrane surface, and (c) the remaining 60–70 residues somewhat disordered but also preferring an orientation parallel to the plane of the membrane.

A better knowledge of the mosaic spread of the sample film is required for a more exact estimate of the helix orientation. An x-ray diffraction measurement on various phospholipid bilayers gave a mosaic spread of $<15°$ (47). In contrast, the presence of salt crystals could cause an increased mosaic spread for our samples. An estimate of the mosaic spread can be obtained by comparing the direction of the DMPC ester carbonyl (C = O) stretch transition moment determined for our samples with that for pure and well oriented DMPC film. The tilt angle for the C = O stretch transition moment of dry DMPC film is reported to be close to 64° (19, 48). From our dichroism measurements of the 1,737 cm^{-1} band, we find the tilt angle to be 59.5°. The discrepancy with the previous measurements of the pure lipid bilayer could arise from a higher mosaic spread in our sample. Taking this increased mosaic spread into account leads to a prediction that the average α-helix tilt will lie in the range between 45.2° and 49.7°. Alternatively, the smaller angle for the C = O transition moment might arise from a perturbation of the ester carbonyl groups by the colicin E1 fragments incorporated into the bilayer.

The membrane bound colicin E1 peptide showed a surprisingly large and rapid hydrogen/deuterium exchange. After 5 min, $\sim 80\%$ of the peptide hydrogen exchange occurs (fast exchanging hydrogens) with $<5\%$ remaining after 72 h (unexchanged hydrogens). In comparison, films of several soluble proteins including lysozyme, ribonuclease, and myoglobin exhibited $<30\%$ rapid exchange of hydrogen and $>40\%$ unexchanged hydrogens (49). Bacteriorhodopsin exhibits over 73% unexchanged hydrogens after 48 h of direct D$_2$O exposure (22). Thus, the result for the membrane bound colicin E1 channel is unusual. To the best of our knowledge, this is the first report of hydrogen/deuterium exchange in an aqueous channel *in situ*.

The cause of the large fraction of rapid H/D exchange for the colicin E1 channel peptide is presently unknown. One explanation is that the existence of the transmembrane channel allows access to residues incorporated in the bilayer resulting in a fast exchange. According to the data discussed above on peptide insertion into the bilayer, at least 70 residues (40%) are inserted as

inferred from the labeling experiments with asolectin vesicles (46). Thus, if the fast exchange fraction involves the noninserted residues at the membrane periphery, it should not exceed 60%. Another possibility is that the peptide groups are electrostatically perturbed by the KCl. For example, it is known that increasing pH causes an increase in the rate and extent of H/D exchange (49).

CONCLUSIONS

In conclusion, our FTIR measurements on the colicin E1 channel forming COOH-terminal fragment indicate a structure which is predominantly α-helical. These helices give rise to an IR dichroism which would be consistent with a portion of the fragment being incorporated into the membrane with a transverse orientation while the remainder is located parallel to the membrane surface. The rapid H/D exchange found for the protein is unique and it remains to be demonstrated if the existence of the aqueous channel and/or perturbation of helices near membrane surface can be responsible for this unusually fast and complete H/D exchange.

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