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TolA: A membrane protein involved in colicin uptake contains an extended helical region

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ABSTRACT **The group A colicins and the DNA of many single-stranded filamentous bacteriophage are able to use combinations of the Tol proteins to gain entrance into or across the membrane of *Escherichia coli*. The TolA protein is a 421-amino acid residue integral membrane protein composed of three domains. Domain I, consisting of the amino-terminal 47 amino acids, contains a 21-residue hydrophobic segment that anchors the protein in the inner membrane. The remaining 374 amino acids, containing the other two domains, reside in the periplasmic space. Domain III, consisting of the carboxyl-terminal 120 residues, is considered to be the functional domain based on the location of the *tolA592* deletion mutation. The internal 262 amino acids comprise domain II, which connects domains I and III together via short regions of polyglycine. It contains a large number of 3- to 5-residue polyalanine stretches, many of which have a repeat of the sequence Lys-Ala-Ala-Ala-(Glu/Asp). Circular dichroism analysis of different portions of TolA show domain II to be predominantly α-helical in structure while domain III contains ≈10% helical structure.**

The two membranes of *Escherichia coli* present a formidable barrier to the import of macromolecules into the cytoplasm (1). Yet macromolecules such as colicins or the DNA of certain filamentous bacteriophages are able to use specific systems for entry into the membrane or cytoplasm. The discovery of bacterial proteins involved in such import systems has been facilitated by the analysis of mutant bacteria unable to take up these macromolecules. For example, certain mutations in the *tolQRAB* gene cluster can render the bacteria insensitive both to infection by the filamentous single-stranded DNA bacteriophage and to the effects of the group A colicins (2–6). Import of these macromolecules into the *tol* mutants is inhibited after the phage or colicin has bound to its bacterial receptor, specific outer membrane proteins in the case of the colicins and the tip of specific conjugative pili for the filamentous bacteriophage. This implies that the import of phage DNA and colicins is at least a two-step process that requires the Tol proteins only after the molecules have interacted with their respective outer membrane receptors.

TolA is one of the Tol proteins necessary for the uptake of all the group A colicins and Tol-dependent phage and, therefore, is thought to play a central role in the import process. TolA is a 44-kDa protein that is tightly associated with the inner membrane (7). Secondary structure predictions suggest that over one-half of the protein appears to be a long uninterrupted α-helix. This helical region contains repeats of the general sequence Lys_{1-2}-Ala_{3-4}-(Glu/Asp). Marqusee and Baldwin (8) have shown that a similar arrangement of these same residues in model peptides results in stable helix formation.

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We present evidence here that TolA contains three domains: (*i*) the N-terminal or first domain anchors the protein to the inner membrane leaving the remainder of the protein in the periplasm, (*ii*) the central or second domain consists of an extended α-helix, and (*iii*) the C-terminal or third domain is the functional portion of the protein. The locations and roles of these domains in the Tol import system are discussed.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. The *E. coli* strain K17(DE3) is K17 (obtained from N. Zinder, Rockefeller University, New York) lysogenized with the λDE3 carrying the inducible gene for T7 RNA polymerase. K17A1(DE3) contains a mini-Tn*10* inserted near nucleotide 200 of the *tolA* gene. It was constructed by P1 transduction from CH4805 (M182 *tolA-4805*::Tn*10*) obtained from D. Vlazny (Department of Biochemistry, Hershey Medical Center, Hershey, PA). λDE3 (9) and plasmids pET3c and pLysS (10) were obtained from F. W. Studier (Brookhaven National Laboratory, Upton, NY). Plasmid pET3clF contains the pUC18 Geneblock upper strand (Pharmacia) polylinker inserted by blunt-end ligation into the unique *Bam*HI site distal to the T7 translation start site (S10). Plasmid pSKL10 contains the *Nru* I 1.7-kilobase (kb) fragment from pTPS306 (6) blunt-end ligated into the T4 DNA polymerase-treated *Pst* I site of the polylinker of pET3clF. This plasmid contains an intact β-lactamase gene and has most of *tolR* and all of *tolA* under the control of the T7 promoter. Plasmid pSKL17 encodes the TolA-II,III protein, which contains amino acids 43–421 of TolA linked to the vector sequences shown in Fig. 2. It was constructed by ligation of the 1.5-kb *Hin*dIII fragment of pSKL10 into the *Bam*HI site of pET3clF after treatment of both with mung bean nuclease. To construct plasmid pSKL18, which encodes TolA-II (see Fig. 2), pSKL17 was cleaved at the *Eco*RV site within *tolA*, digested with slow BAL-31, and treated with T4 DNA polymerase. The 5.2-kb fragment was isolated from agarose and recircularized, and the transformants were analyzed by DNA sequencing. Plasmid pSKL19, which encodes TolA-III (see Fig. 2), was constructed by cutting pSKL10 with *Bam*HI and *Sst* I and removing nucleotides from the *Bam*HI site into the *tolRA* insert by treatment with *Exo* III. S1 nuclease and Klenow were used to blunt end the DNA, and the remaining fragment was ligated together.

Media and Chemicals. Bacteria were routinely grown as described by Sun and Webster (5). Isopropyl β-D-thiogalactopyranoside (IPTG) was obtained from Research Organics; ^{125}I-labeled protein A was purchased from DuPont; RNase A was obtained from Millipore; and egg white lysozyme, trypsin, and trypsin inhibitor were from Sigma. Restriction endonucleases and DNA modifying enzymes were purchased from Boehringer Mannheim and Bethesda Research Laboratories.

Abbreviation: IPTG, isopropyl β-D-thiogalactopyranoside.

Protein Purification. *E. coli* K17(DE3) cells containing pLysS and either pSKL17, -18, or -19 were grown to an OD_{600} of 0.5 at 37°C and induced with 0.4 mM IPTG for 2 hr. The cells were harvested; washed with 0.1 M Tris·HCl (pH 8); resuspended in the same buffer containing 0.5 mM EDTA, lysozyme (100 μg/ml), and RNase A (20 μg/ml); and incubated at 0°C for 20 min. Breakage of the cells was completed by sonication and an S100 supernatant was obtained by centrifugation at 106,000 × *g* for 3.5 hr. The proteins were precipitated from the supernatant by using ammonium sulfate in the following ranges of saturation: TolA-II,III, 40–50%; TolA-II, 30–50%; TolA-III, 50–70%. The pellets were resuspended in 10 mM Tris·HCl (pH 7.4) and dialyzed into the same buffer, and the sample was applied to a DE52 column (2 × 14 cm) previously equilibrated with the dialysis buffer. The TolA proteins, which are not adsorbed to DE52, were collected and adsorbed to a Mono S column (Pharmacia) equilibrated with 10 mM Tris·HCl (pH 7.4) and eluted with a linear NaCl gradient. TolA-II,III eluted at 0.08 M NaCl, TolA-II eluted at 0.08 M, and TolA-III eluted at 0.18 M. The proteins were analyzed for purity by SDS/PAGE by the procedure of Laemmli (11) and determination of the amino acid composition and N-terminal sequence. Rabbit antibodies to TolA were obtained by subcutaneous injection of purified TolA-II,III in complete Freund's adjuvant. The IgG used for Western blot analysis was purified from the serum by chromatography on protein A-Sepharose (Pharmacia) and stored in phosphate-buffered saline (0.01 M NaP_i/0.15 M NaCl).

Cellular Fractionations and TolA Localization. Periplasmic material was isolated by the osmotic shock procedure (12) in the presence of 0.5 mM EDTA. After incubation with lysozyme (100 μg/ml) for 20 min, the shocked cells were lysed by passage through a 27-gauge needle in the presence of 1 M NaCl (10 μg/ml). Unbroken cells were removed by centrifugation at 4000 × *g* for 15 min and the supernatant was subjected to centrifugation at 130,000 × *g* for 3 hr to separate the cytoplasmic fraction (supernatant) from the membrane fraction (pellet). The periplasmic and cytoplasmic proteins were concentrated by precipitation from 5% trichloroacetic acid. The proteins were solubilized, separated on 10% SDS/polyacrylamide gel, transferred to nitrocellulose, and subjected to Western blot analysis using the anti-TolA-II,III antibody and ^{125}I-labeled protein A as a probe (7).

To analyze the regions of TolA present in the periplasm, cells were incubated at 0°C for 10 min in 8.55% (wt/vol) sucrose/1 mM EDTA/0.03 M Tris·HCl, pH 8. The cells were centrifuged and resuspended in the same solution minus EDTA and equal aliquots were treated with trypsin (25 μg/ml) or buffer at 14°C for 1 hr. Trypsin inhibitor was added to 25 μg/ml and the cells were centrifuged and resuspended in sample buffer. The proteins were separated by SDS/PAGE and subjected to Western blot analysis using antibody against TolA-II,III as described above.

Spectroscopy. CD was examined between 4°C and 40°C with a Jovin-Ybon dichrograph (mark V) interfaced to an Apple IIe computer. This instrument was calibrated with *d*-10-camphorsulfonic acid whose $[\theta]_{290.5}$ = 7800 deg·cm^2·dmol^{-1} and $[\theta]_{192.5}/[\theta]_{290.5}$ = −1.90 (13). Samples were equilibrated at the indicated temperature for 15 min prior to data collection. Data were taken at 0.1-nm intervals, corrected for buffer baseline, and represent the average of three scans. Data are expressed as mean residue molar ellipticity, $[\theta]$, based on the mean residue weights calculated from the appropriate amino acid sequences. Protein concentrations in the stock solutions were determined either by quantitative amino acid analysis (described below) or the microbiuret procedure (14) using bovine serum albumin ($A_{280}^{0.1\%}$ = 6.27 cm^{-1}) (15) as a standard in the latter procedure. These two quantitation procedures differed from each other by ±7%. Gravimetrically calibrated micropipettes were used in making these and all subsequent described dilutions from stock solutions.

Amino Acid Analysis and Sequence. Lyophilized samples were hydrolyzed by exposure to the vapor released from 6.0 M HCl at 115°C for 24 hr in a fan-forced oven (16). Norleucine was added as an internal standard. Based on the sequence of the TolA protein (7), the amount of protein was calculated from the amount of the stable amino acids in the hydrolyzate; results for serine, threonine, tryptophan, tyrosine, cysteine, and methionine were not used. Precolumn modification of amino acids using phenylisothiocyanate (PITC) and HPLC separation of the resulting PITC amino acid derivatives were performed essentially as described (17). N-terminal sequences were determined with a Porton 2090 integrated sequencing system equipped with a Hewlett–Packard 1090 HPLC for on-line analysis of the phenylthiohydantoin derivatives.

RESULTS

Isolation of the Predicted Functional Domains of TolA. The sequence of *tolA* predicts that this gene encodes a 421-amino acid protein of 44.2 kDa (7). Genetic data together with

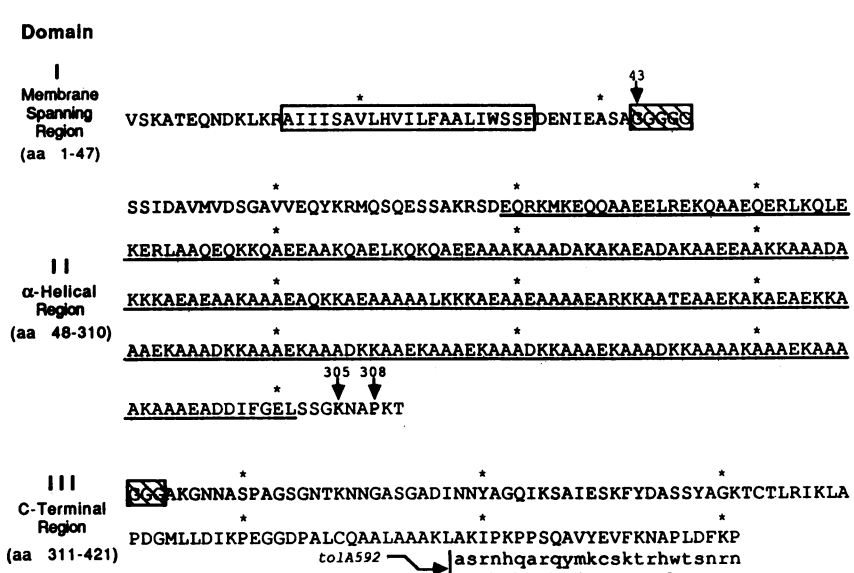

FIG. 1. Predicted amino acid sequence of the TolA domains. The TolA amino acid sequence is divided into three domains with the residues contained in each noted at the left. Open box in domain I encloses the hydrophobic membrane-spanning region. Hatched boxes enclose polyglycine regions. Domain II sequences underlined are those predicted to be α-helical. The position of the *tolA592* deletion with the resulting change in sequence is shown below the wild-type sequence in domain III. * marks every 20th amino acid (aa).

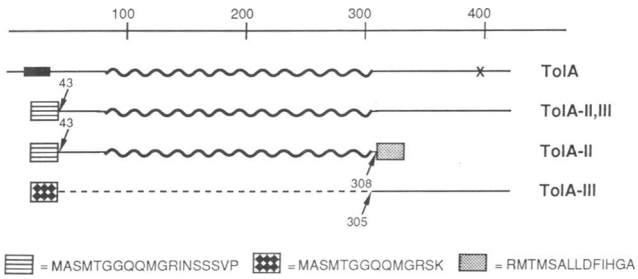

FIG. 2. Schematic drawing of truncated TolA proteins. Scale at the top represents amino acid position. The specific start and stop of each protein is indicated. X in TolA signifies the position of the *tolA592* mutation. TolA-II,III is encoded in pSKL17 and contains amino acids 43–421 of TolA (see Fig. 1). TolA-II is encoded in pSKL18 and has amino acids 43–308 of TolA, while pSKL19 encodes TolA-III containing residues 305–421 of TolA. The vector sequences in each truncated protein are noted below the figure. ▬▬, Hydrophobic membrane spanning region; ∼∼, predicted helical region; ------, deletion of the region.

secondary structure predictions of the protein sequence suggest that the protein is composed of the three domains shown in Fig. 1 (7). The N-terminal 47 residues contain a hydrophobic segment of 21 amino acids, which could span the inner membrane and anchor TolA in it. The second domain between amino acids 48 and 310 is predicted to contain a 223-residue uninterrupted α-helical region. The remaining C-terminal residues constitute the third domain, thought to be functionally important since mutant *tolA592* has a 4-bp deletion leading to the alternative reading frame shown in Fig. 1 (7). Separating the domains are polyglycine regions possibly required as hinges between the domains.

To further analyze the location and structure of these regions, various portions of the *tolA* gene were cloned into the pET3c expression vector, and the proteins were overproduced and purified. As shown in Fig. 2, TolA-II,III contains domains II and III of TolA, TolA-II contains domain II, and TolA-III contains domain III. A few non-TolA residues are present at the beginning and end of these proteins due to the translation of vector sequences (Fig. 2). Each protein was at least 95% pure as judged by SDS/PAGE and comparison of the expected and observed amino acid analysis of each protein (data not shown). Purified TolA-II,III and TolA-II were subjected to 14 cycles of N-terminal sequence analysis and the results were consistent with 95% or greater NH_2 purity for both proteins. Unexpectedly, we found that TolA-II,III was processed with methionine as the N-terminal amino acid, whereas TolA-II contained alanine as the N-terminal amino acid.

Rabbit antibodies against TolA-II,III are able to recognize TolA in a wild-type cell, but not in the K17A1(DE3) construct with mini-Tn*10* inserted in the *tolA* gene (Fig. 3*A*, compare lanes 1 and 2). The amount of radioactivity in the TolA band from wild-type cells was compared to the amount of radioactivity of various concentrations of purified TolA-II,III included in the same Western blot. We estimate that a logarithmically growing culture contains between 400 and 800 molecules of TolA.

The Cellular Localization of TolA. To determine whether the N-terminal domain of TolA is in the membrane, the cellular location of TolA and each of the truncated proteins shown in Fig. 2 was analyzed. Bacteria containing the appropriate plasmid were induced to produce a particular TolA protein by the addition of IPTG. The periplasmic, cytoplasmic, and membrane fractions were analyzed for the presence of TolA proteins by Western blot analysis using antibodies against TolA-II,III. Only the intact TolA protein was associated with the membrane fraction (Fig. 3*B*, lane 3), while all three of the truncated proteins were detected exclusively in the cytoplasmic fraction (lanes 10, 14, and 18). Some cleavage of intact and truncated proteins was observed to occur during cellular fractionation (compare lanes WC and C).

To analyze whether any other portion of TolA was membrane associated, EDTA-permeabilized bacteria producing TolA or TolA-II,III were treated with trypsin and the remaining bacterial proteins were probed for the presence of TolA by Western blot analysis (Fig. 3*C*). Trypsin treatment removed all antibody reactive material in cells overproducing TolA (Fig. 3*C*, lane 22), suggesting that all of the TolA protein distal to the hydrophobic region resides in the periplasm. This

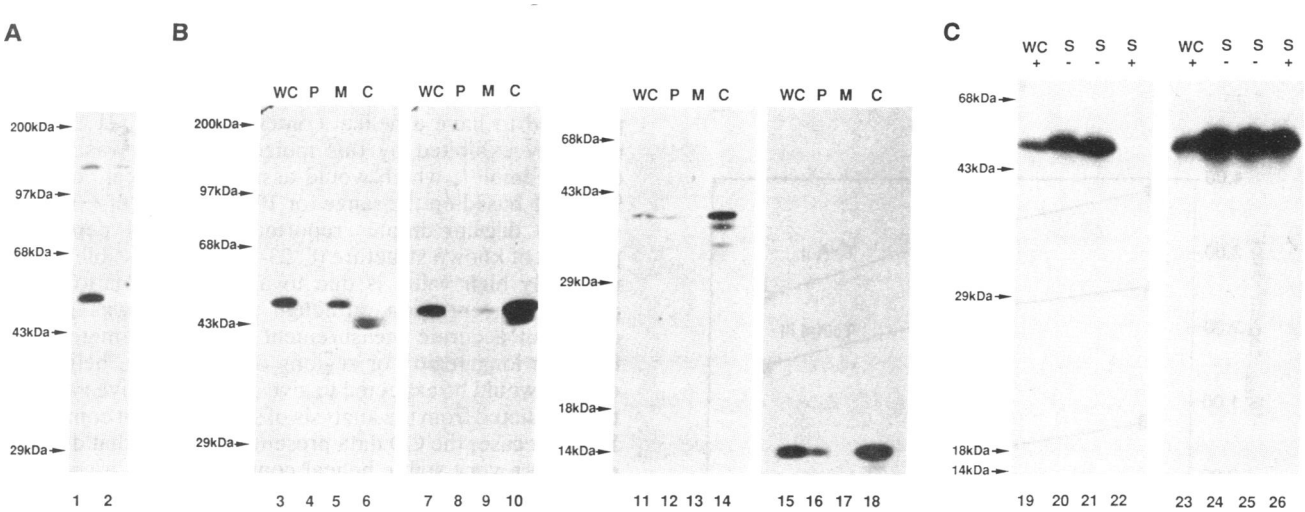

FIG. 3. Localization studies of TolA and truncated TolA proteins. Exponentially growing cells containing the appropriate plasmids were induced with IPTG and solubilized in sample buffer, and the proteins were subjected to Western blot analysis using anti-TolA-II,III IgG and ^{125}I-labeled protein A as a probe. (*A*) Lanes: 1, K17(DE3) whole cells; 2, K17A1(DE3) whole cells. (*B*) Membrane fractionations were performed as described in *Materials and Methods*. WC, whole cells; P, periplasm; M, membrane; C, cytoplasm. Lanes: 3–6, K17(DE3)/pSKL10 encoding wild-type TolA; 7–10, K17(DE3)/pSKL17 encoding TolA-II,III; 11–14, K17(DE3)/pSKL18 encoding TolA-II; 15–18, K17(DE3)/pSKL19 encoding TolA-III. (*C*) Trypsin experiments were performed as described in *Materials and Methods*. Lanes: 19–22, K17(DE3)/pSKL10 encoding wild-type TolA; 23–26, K17(DE3)/pSKL17 encoding TolA-II,III. WC, whole cells; S, EDTA solubilized cells; +, trypsin added to cells; −, no trypsin added to cells. Lanes 20 and 24 show the 0-min time point of EDTA-solubilized cells. All other lanes show the results from cells that were incubated at 14°C for 1 hr in the presence or absence of trypsin.

region includes both domains II and III as the antibody would have detected these regions (Fig. 3*B*, lanes 14 and 18). The TolA-II,III protein, which is contained exclusively in the cytoplasm, was not digested by trypsin (Fig. 3*C*, lane 26). These data show that the N-terminal region, which contains the 21-amino acid hydrophobic sequence, must act as both the signal sequence and the only membrane anchor for TolA.

CD. Fig. 4*A* shows the far UV CD spectra for TolA-II,III, TolA-II, and TolA-III at 4°C taken in 20 mM potassium phosphate buffer (pH 7.4) containing 0.15 M potassium fluoride. The signal was corrected for the small differences in mean residue weights for each of the proteins, which were calculated to be 101.0 for TolA-II,III, 102.0 for TolA-II, and 101.7 for TolA-III. The unusually high percentage of helical structure present in TolA-II,III and TolA-II is supported by the large magnitude of the 222-nm minimum, the position of zero ellipticity (200 nm), and the intensity and position of the single positive CD transition at 190 nm (13). As predicted from the amino acid sequence, TolA-II contains the greatest degree of helical structure ($[\theta]_{222} = -38{,}500$ deg·cm^2·dmol^{-1}). TolA-III exhibits only a moderate helical content ($[\theta]_{222} = -7300$ deg·cm^2·dmol^{-1}), while TolA-II,III, containing both regions, has the expected intermediate helical content ($[\theta]_{222} = -25{,}500$ deg·cm^2·dmol^{-1}). Both of the TolA peptides, TolA-II and TolA-II,III, which have the predicted extended helical region, were quite stable to thermal unfolding since a temperature change from 4°C to 40°C resulted in only a 35–40% decrease in $[\theta]_{222}$ (Fig. 4*B*).

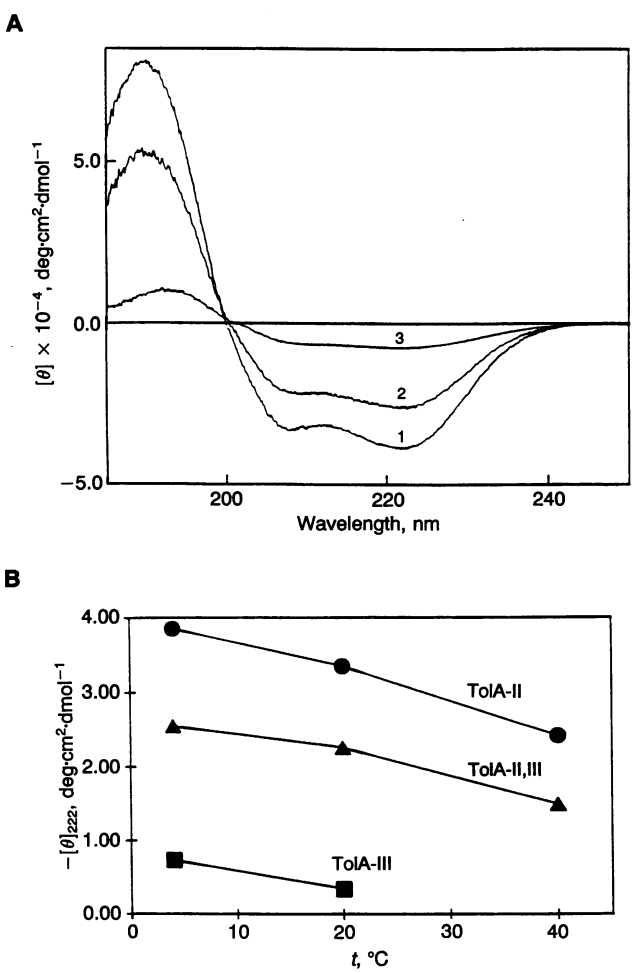

FIG. 4. CD studies of TolA and truncated TolA proteins. (*A*) Comparison of the far UV CD spectra of TolA-II,III (trace 2, 43 μg/ml), TolA-II (trace 1, 47 μg/ml), and TolA-III (trace 3, 209 μg/ml). The CD spectra were recorded at 4°C and are expressed as described in *Materials and Methods* and *Results*. (*B*) Temperature dependence of the magnitude of the 222-nm peak (diagnostic of α-helical character) for TolA-II,III (▲), TolA-II (●), and TolA-III (■). Conditions are the same as in *A*.

Molar ellipticity values at 222 nm representative of 100% helical character range from $-35{,}700 \pm 2800$ deg·cm^2·dmol^{-1} for the extended helix poly(L-lysine) (18) to $-32{,}600 \pm 1900$ deg·cm^2·dmol^{-1} computed for a limited set of representative proteins whose structures have been determined from x-ray crystallographic studies (19). A recent compilation of reference CD spectra reports a value of $-37{,}400$ deg·cm^2·dmol^{-1} for 100% helical conformation based on an average value of 10.4 residues per helical segment (13). The length of the helix influences the magnitude of the signal at 222 nm (20), and in general the magnitude of the signal decreases with decreasing chain length. Yang *et al.* (13) have calculated $[\theta]_{222} = -40{,}000$ deg·cm^2·dmol^{-1} for a 40-residue helix. Assuming that the helical structure in TolA is uninterrupted, we can use this value to estimate the degree of helical structure to be >90% in the construct TolA-II. No matter what model is used to estimate the percentage helical content, the data presented here firmly demonstrate that the TolA protein possesses a remarkable extent of helical structure.

DISCUSSION

The data presented here are consistent with the proposed three-domain structure for TolA depicted in Fig. 1. The N-terminal 42 amino acids must act as both a signal and stop transfer domain since removal of this region results in the protein being confined to the cytoplasm. The residues distal to amino acid 34 are translocated to the periplasm and remain anchored to the inner membrane, presumably via the hydrophobic stretch of 21 amino acids between residues 14 and 35. The C-terminal 120 amino acids, designated domain III, are required for function based on the location of the deletion in *tolA592* (4, 7). Separating domain III from domain I is a long sequence of amino acids that is extremely rich in α-helical structure. Between the three domains are short stretches of glycine (see Fig. 1), which possibly act as flexible hinge regions.

Domain II is unusual in a number of aspects. It contains a 223-amino acid sequence (Fig. 1, residues 79–301) that has a very high percentage of alanine, lysine, glutamic acid, and aspartic acid and is predicted to be entirely α-helical. TolA-II, which is composed almost exclusively of this domain, was predicted to have a helical content of ≈87% (21, 22). The ellipticity exhibited by this molecule at 4°C was $-38{,}500$ deg·cm^2·dmol^{-1}, which would assign it a helical content of 90–100% based on the range for 100% helicity of $-36{,}000$ to $-40{,}000$ deg·cm^2·dmol^{-1} reported for model peptides or proteins of known structure (8, 23–25). It is possible that this unusually high value is due to an underestimation of the protein concentration, although great care was given to obtain an accurate measurement of this parameter. More likely, a long region or regions of continuous helix in the domain would be expected to give a more negative value than that predicted from the analysis of shorter model compounds. In either case, the CD data presented confirm that domain II exists in a very stable helical conformation at a reasonable physiological salt concentration. Its sequence contains all the hallmarks expected for a helical protein. Alanine comprises almost 45% of the amino acid composition of domain II and most of it is arranged in polymeric stretches of three or four residues, consistent with the observation that short alanine peptides show stable helix formation (26). In addition, 14 of the alanine regions have the sequence Lys-Ala$_3$-(Glu/Asp), in which the lysine and glutamic acid residues have been shown to form stable helices through salt bridge interactions (8, 27, 28).

The CD spectra are consistent with the prediction that domain II consists of one or more regions of continuous helix. Although the helix would contain a net positive charge, the arrangement of the charged residues in the sequence would present a relatively equal distribution of both charged species along the length of this region. One can only speculate as to the role of this domain in the normal function of TolA. It may play the role of a tether with flexible ends, allowing the functional third domain to interact with colicin/phage receptor protein complexes in the outer membrane while remaining anchored to the inner membrane via domain I. Further studies are necessary to determine whether domain II is one continuous helix or contains bends or turns not predicted by its amino acid sequence.

The difficulty in assessing the role of TolA in the uptake of macromolecules stems partly from the fact that there is no known role for this protein in the bacterium. It may not normally be involved in the uptake of macromolecules but it may perform some entirely different function for the cell. Mutations in *tolA* as well as *tolQ*, *-R*, and *-B* appear to grow normally, although they do show increased sensitivity to detergents, suggesting that they play some role in maintaining the integrity of the membrane (2–6). These proteins may be involved in the assembly of the outer membrane, transporting molecules from the cytoplasm through the periplasm to a position where they can be incorporated into the outer membrane. The role of TolA in such a mechanism could be to bring the inner and outer membranes together via its three-domain structure and thus provide a direct link between the two membranes. Such bridges or "adhesion zones" have been proposed to exist (29) but the exact nature of such a structure is not known (30). The group A colicins and the filamentous bacteriophage may have evolved to take advantage of such a structure to gain entry into the cell. Determining the mechanism by which TolA and the other Tol proteins allow colicin and phage DNA translocation may also assist in deciphering the normal role of TolA in the cell. In addition, such knowledge should give a clearer picture of how macromolecules are transported across biological membranes into a living cell.

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