Indolicidin, an antimicrobial tridecapeptide-amide isolated from the cytoplasmic granules of bovine neutrophils (1), exhibits its activity against a variety of Gram-positive and Gram-negative bacteria, as well as fungi. Its primary mode of action appears to be membrane permeabilization (2, 3). Previously we demonstrated that indolicidin binds to both charged and neutral lipid membranes and causes the leakage of their contents (3). Indolicidin’s amino acid content is quite remarkable because of its five tryptophans and three proline residues. It is a member of the cathelicidin gene family and is often classified as a member of the proline-rich antimicrobial peptides (4), but its sequence is markedly more hydrophobic than the other members of this family. Several studies, based upon NMR and FTIR measurements, suggested polyproline II helix as a structural motive for other proline-rich peptides, such as PR-39 (5) and bactenecin 5 (6). However, in both of these cases, evidence for polyproline II helix by CD spectroscopy was inconclusive. Falla et al. (7) concluded from CD data alone that an indolicidin variant lacking the C-terminal amide adopted a “much stronger poly-L-proline II structure” upon membrane binding than PR-39 or bactenecin 5. In contrast, we reported CD spectra of indolicidin in various solvents and when bound to micelles (5), but noted a sharp negative band at 227 nm arising from the tryptophan residues, that would mask spectral features characteristic of poly-L-proline II helix.

We have reexamined this issue by means of CD measurements of native indolicidin and several of its analogues. None of the features characteristic of a poly-L-proline helix (or α- or 3α-helix) were observed for any of the peptides studied. To eliminate artifacts associated with tryptophan, we synthesized indolicidin-L and indolicidin-F in which all five tryptophans were replaced with leucines or phenylalanines, respectively. The changes in CD spectra of these Trp-free peptides upon transfer into membrane-like environments were found to be consistent with the formation of β-turns. For the native indolicidin in SDS micelles, temperature increases resulted in a coupled diminution of two sharp bands, a negative one at 227 nm and a positive one at 217 nm. This phenomenon, which is absent in indolicidin-L variants with single Leu→Trp substitutions, is consistent with exciton splitting produced by the stacking of indole rings. Type VI turns in model peptides in aqueous solution are known to be promoted by stacking interactions between cis-proline and neighboring aromatic residues [Yao et al. (1994) J. Mol. Biol. 243, 754]. Molecular modeling of indolicidin with a Trp6-cis-Pro7-Trp8 type VIa turn demonstrated the feasibility of this turn conformation and revealed the possibility of an accompanying amphipathic structure. We therefore suggest that turn conformations are the principal structural motif of indolicidin and that these turns greatly enhance membrane activity.

ABSTRACT: Indolicidin is a 13-residue antimicrobial peptide-amide isolated from the cytoplasmic granules of bovine neutrophils that contains five Trp and three Pro residues. Falla et al. [(1996) J. Biol. Chem. 271, 19298] suggested that indolicidin forms a poly-L-proline II helix based upon the circular dichroism (CD) spectra of a closely related peptide (indolicidin methyl ester). In contrast, we found no evidence of poly-L-proline II helix formation in the CD spectra of native indolicidin in various solvents or when bound to micelles and membranes [Ladokhin et al. (1997) Biophys. J. 72, 794]. We interpreted the spectra as arising from unordered and/or β-turn structures, but noted a sharp negative band at 227 nm arising from the tryptophan residues that would mask spectral features characteristic of poly-L-proline II helix. We have reexamined this issue by means of CD measurements of native indolicidin and several of its analogues. None of the features characteristic of a poly-L-proline helix (or α- or 3α-helix) were observed for any of the peptides studied. To eliminate artifacts associated with tryptophan, we synthesized indolicidin-L and indolicidin-F in which all five tryptophans were replaced with leucines or phenylalanines, respectively. The changes in CD spectra of these Trp-free peptides upon transfer into membrane-like environments were found to be consistent with the formation of β-turns. For the native indolicidin in SDS micelles, temperature increases resulted in a coupled diminution of two sharp bands, a negative one at 227 nm and a positive one at 217 nm. This phenomenon, which is absent in indolicidin-L variants with single Leu→Trp substitutions, is consistent with exciton splitting produced by the stacking of indole rings.

Type VI turns in model peptides in aqueous solution are known to be promoted by stacking interactions between cis-proline and neighboring aromatic residues [Yao et al. (1994) J. Mol. Biol. 243, 754]. Molecular modeling of indolicidin with a Trp6-cis-Pro7-Trp8 type VIa turn demonstrated the feasibility of this turn conformation and revealed the possibility of an accompanying amphipathic structure. We therefore suggest that turn conformations are the principal structural motif of indolicidin and that these turns greatly enhance membrane activity.

1 This research was supported by Grants GM-46823 (S.H.W.) and AI-22931 (M.E.S.) from the National Institutes of Health.
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We then examined the CD data of our indolicidin-based peptides for evidence of the polyproline helix signal and considered the issue of the detectability of a hypothetical polyproline CD signal from natural indolicidin, given the complications presented by five tryptophan residues. Finally, we examined the CD spectrum of an indolicidin analogue that lacked aromatic residues. Taken together, our data indicated that indolicidin peptides do not exhibit the features expected of polyproline helices. However, the data from native indolicidin suggested a conformation that involves the stacking of indole rings. Molecular modeling suggested that such stacking can be achieved by a type VIa turn conformation that is known (8) to be promoted by -aromatic-Pro-aromatic- amino acid sequences such as those found in indolicidin. We therefore conclude that turn conformations, rather than polyproline helix, are the main structural motif of indolicidin peptides.

MATERIALS AND METHODS

Materials. Lipids were obtained from Avanti Polar-Lipids (Alabaster, AL), and SDS and poly-L-proline of MW 4800 (by viscosity) were from Sigma (St. Louis, MO). The buffer was a 10 mM potassium phosphate solution, pH 7.0.

Peptides were synthesized using standard Fmoc chemistry and purified by the method of Van Abel et al. (9). After purification, the peptides were >98% pure as judged by analytical HPLC, electrophoresis, and mass spectroscopy. The following peptides were synthesized for this study:

The following peptides were synthesized for this study:

Native indolicidin (Indolidicidin-W):
Ile-Leu-Pro-Trp⁶-Lys-Trp⁶-Pro-Trp⁶-Pro-Trp⁶-Pro-Trp⁶-Arg-Arg-Amide
Indolidicidin-L:
Ile-Leu-Pro-Leu⁴-Lys-Leu⁶-Pro-Leu⁶-Leu⁸-Pro-Leu⁶-Arg-Arg-Amide
Indolidicidin-F:
Ile-Leu-Pro-Phe⁴-Lys-Phe⁶-Pro-Phe⁶-Pro-Phe⁶-Pro-Phe⁶-Arg-Arg-Amide
Indolidicidin-L-W⁶:
Ile-Leu-Pro-Trp⁶-Lys-Leu-Pro-Leu-Pro-Leu-Ala-Ala-Amide
Indolidicidin-L-L⁶:
Ile-Leu-Pro-Leu-Ala-Pro-Leu-Pro-Leu-Pro-Leu-Ala-Ala-Amide
Indolidicidin-L-W⁶:
Ile-Leu-Pro-Lys-Leu-Pro-Trp⁶-Leu-Pro-Leu-Pro-Leu-Ala-Ala-Amide

In the three single-tryptophan-containing analogues, above, Arg¹² was replaced with Ala in order to restore some of the hydrophobicity lost due to the Trp-to-Leu substitution (10). A similar Arg→Ala substitution in indolidicidin-W did not affect the CD spectrum (data not shown).

Preparation of Vesicles. Large unilamellar vesicles (LUV)¹ approximately 0.1 μm in diameter were formed by extrusion under N₂ pressure through Nucleopore polycarbonate membranes using the method of Mayer et al. (11).

Circular Dichroism and Absorbance Spectroscopy. CD measurements were performed using a Jasco-720 spectropolarimeter (Japan Spectroscopic Co., Tokyo). In the far-UV region (185–260 nm), 20–200 scans were recorded, while in the near-UV region (260–330 nm) 500 scans were recorded. A 1 mm optical path was used for far-UV, and 10

¹ Abbreviations: POPG, palmitoyloleoylphosphatidylglycerol; LUV, large unilamellar vesicles (100 nm diameter).

RESULTS

The CD spectrum of poly-L-proline in aqueous solution is presented in Figure 1. This spectrum reproduces well those published in the literature (13) and has the two essential features of a polyproline II helix: a strong negative band at 205 nm and a weak positive band at 230 nm. The inset presents unnormalized data measured with three poly-L-Pro samples at concentrations of 1.0, 0.35, and 0.03 mg/mL. These data demonstrate clearly the characteristic positive band at 230 nm for near-UV. UV absorbance was measured with a Cary 3E spectrophotometer (Varian Analytical Instruments, Sugar Land, TX). CD spectra were found to be independent of concentration in the range of 5–30 μM for indolidicidin-W and up to 100 μM for the other peptides.

Molecular Modeling. Molecular models were created using Swiss Pdb Viewer 3.1 (http://www.expasy.ch/spdbv). The -Trp⁶-cis-Pro⁷-Trp⁶- turn was set according to a type VIa turn (12) with tryptophan side chains arranged to pack around the proline ring. The rest of the molecule was set arbitrarily. The final model had no backbone or side chain clashes and had Φ,Ψ angles within the allowed regions of the Ramachandran diagram.

Figure 1: CD spectrum of poly-L-Pro in aqueous solution. This spectrum reproduces well those published in the literature (13) and has the two essential features of a polyproline II helix: a strong negative band at 205 nm and a weak positive band at 230 nm. The inset presents unnormalized data measured with three poly-L-Pro samples at concentrations of 1.0, 0.35, and 0.03 mg/mL. These data demonstrate clearly the characteristic positive band at 230 nm.
or in membrane-like environments are dramatically different (Figure 2A and Figure 3A). Two relatively broad bands at 197–200 and 227–230 nm are present in all of the spectra, but membrane binding or transfer to trifluoroethanol results in increases of the 227–230 nm band at the expense of the 197–200 nm band. Thus, the difference spectra obtained by subtracting the spectrum for indolicidin-L in buffer from the corresponding spectrum in TFE or POPG. The difference spectra are consistent with the formation of a $\beta$-turn (see text).

![CD Spectra of Indolicidin Antimicrobial Peptides](image)

**Figure 2:** CD spectra of indolicidin-L in various media. (A) Indolicidin-L in aqueous buffer (solid line), bound to POPG (dashed line), and in trifluoroethanol (dotted line) exhibit bands at 197–200 and 227–230 nm. Membrane binding and transfer to trifluoroethanol result in a similar increase of the latter band at the expense of the former (see text). (B) Difference CD spectra obtained by subtracting the spectrum for indolicidin-L in buffer from that in POPG (Figure 2B, dashed line) or TFE (Figure 2B, dotted line) have a positive and a negative band. This is consistent with the formation of a $\beta$-turn with the B-type topology predicted by Woody (14) and observed in cyclic peptides by Deslauriers et al. (15).

Remarkably, heating of indolicidin-L in SDS (Figure 3A) results in a transition similar to that for membrane binding (compare to Figure 2A). This behavior, also observed for indolicidin-L in aqueous environment (data not shown), is not understood. It may reflect the inherent temperature dependence of the CD spectrum of the unfolded state (see Discussion). Similar behavior is observed for indolicidin-F (Figure 3C).

Although the CD spectrum of aqueous indolicidin-W has a single negative band with a minimum at about 202 nm as observed for indolicidin-L, a new sharp negative band appears at 227 nm upon partitioning into membranes and micelles (3). The temperature dependence of the CD spectrum in SDS shown in Figure 3B discloses that the negative band at 227 nm is coupled to the peak at 217 nm that separates the minima seen at 202 and 227 nm. This behavior is that expected from exciton splitting produced by aromatic chromophores stacking against one another (16–18). The CD spectra for indolicidin-F (Figure 3C) appear to have features of both indolicidin-L and indolicidin-W. The principal negative band is at 202 nm as for indolicidin-W, but the overall temperature dependence is reminiscent of indolicidin-L, suggesting that the plausible exciton couplet between Phe residues is much weaker than for Trp residues. This is to be expected because of the lower oscillatory strength of Phe. In any case, Phe may contribute to the overall appearance of the CD spectrum in the far-UV region.

The CD spectra of single-Trp indolicidin variants in buffer (solid lines) and in SDS micelles (dashed lines) are presented in Figure 4. These variants, with tryptophan in positions 4, 8, and 11 (Figures 4A, 4B, and 4C, respectively), behaved similarly to indolicidin-L but not indolicidin-W (compare to Figure 3). This suggests that the collective properties of the multiple tryptophans of indolicidin-W are responsible for its peculiar CD spectrum. Importantly, none of these single-Trp variants showed evidence of the presence of poly-L-proline helices (compare to Figure 5).

The strong exciton couplet observed for the $B_\alpha$ transition of tryptophan in indolicidin-W in SDS (Figure 3B) raised
the possibility of observing a similar splitting in the near-UV region. However, as pointed out by Grishina and Woody (17), the strength of the couplet in this region is likely to be 50 times weaker because it depends on the square of the oscillator strength and hence extinction. Additional complications arise from the complexity of the tryptophan absorbance band in the near-UV, consisting of two overlapping transitions, \( \lambda_a \) and \( \lambda_b \), each with different orientation, vibrational structure, and sensitivity to the polarity of the environment. The unambiguous identification of the exciton couplet in the near-UV thus seems unlikely. This is confirmed in Figure 6 by the near-UV spectrum for indolicidin-W (solid line) that is virtually identical in shape to the spectrum of indolicidin-L-W (dashed line) which can have no exciton couplet. We conclude, as expected from general principles, that the near-UV region is not useful for identification of exciton couplets.

**DISCUSSION**

CD spectroscopy is a well-established tool for studying the secondary structure of proteins and peptides. Its high sensitivity allows the conformational changes induced by the changes in environment to be monitored. Such changes are frequently observed when peptides which are largely unstructured in solution partition into lipid bilayers (19). In those cases, so-called partitioning–folding coupling (10) induces secondary structure formation because hydrogen bonding reduces the energetic cost of partitioning peptide bonds (20, 21). The CD spectrum of indolicidin also changes upon partitioning into bilayers (3, 7), but the interpretation of the spectrum of indolicidin in terms of secondary structure formation is problematic because of its unusual composition. Given indolicidin’s high proline content, one might assume that the peptide forms a polyproline helix upon partitioning into a lipid environment (7). The data presented here, as discussed below, are not consistent with that interpretation, however. The CD spectrum of a polyproline helix, as confirmed in Figure 1, characteristically has a strong negative band in the vicinity of 200 nm and a weak positive band at 230 nm (22). The latter band is generally considered as uniquely indicative of this conformation because thermal denaturation of collagens usually leads to complete loss of the positive band with only minor changes in the position of the negative band (22). The latter band is generally considered as uniquely indicative of this conformation because thermal denaturation of collagens usually leads to complete loss of the positive band with only minor changes in the position of the negative band (22). The negative band observed at 205 nm is not unique to the polyproline helix. Similar bands are observed for random coil conformations and for \( \beta \)-turns which are predicted to exhibit a wide range of CD patterns (14).

None of the CD spectra presented here of indolicidin or its variants (Figures 2–4) show the characteristic positive band of poly-L-proline at 230 nm (Figure 1). Based upon IR spectra, Bahng et al. (23) recently proposed that indolicidin...
forms a $\beta$-helix when bound to membranes, but our CD spectra do not support that idea. Their measurement of the FTIR-ATR spectra of indolicidin dried on a ZnSe crystal or in a lipid bilayer showed a maximum of 1664 cm$^{-1}$ accompanied by significant broadening that can also be explained by other types of secondary structure, e.g., reverse turns (24). The authors justified their $\beta$-helix assignment by reproducing our earlier CD result for membrane-bound indolicidin (3) that had none of the spectral features expected of an $\alpha$-helix. Because the CD appearance of a $\beta$-helix is very close to that of an $\alpha$-helix (25, 26), the assignment of the spectrum as that of a $\beta$-helix is not justified. The main difficulty in interpreting the CD spectrum of indolicidin is the interference caused by the tryptophan residues in the region where the positive band of the polyproline helix is expected to appear (compare Figures 1 and 3B). Tryptophan residues, and other aromatic residues, are expected to contribute to CD spectra in the far-UV region that is associated with the peptide bond. This conclusion is based on the calculations discussed by Woody (27) and studies of gramicidin (28) and other tryptophan-containing membrane-binding peptides (20). As a result, we attributed the appearance of the negative peak at 227 nm in the CD spectrum of indolicidin partitioned into membranes and micelles to tryptophan side chains (3). This assignment is confirmed by the temperature-dependent changes of the CD spectrum of indolicidin-W in SDS micelles (Figure 3B). The observed coupling of the negative peak at 227 nm with the positive peak at 217 nm is characteristic of the stacking of aromatic rings (16–18). Such stacking is expected to be most efficient when aromatic residues are separated by proline, as they are in indolicidin. This follows from the efficiency of tryptophan cross-linking in various peptides reported by Stachel et al. (29). Tryptophans in indolicidin-W will also form such cross-links under certain conditions (Selded et al., unpublished), suggesting the possibility of a conformation in which tryptophans come together in some kind of turn conformation.

If the prolines of indolicidin were to promote the polyproline I helix conformation of the backbone, then this should be apparent in the CD spectra of indolicidin-L. As Figures 2 and 3A demonstrate, there is no evidence of the characteristic positive peak of a polyproline helix at 230 nm. However, the spectrum is also not characteristic of simple random-coil conformations because of the strong negative peak at 230 nm. Although $\beta$-turns are generally difficult to identify by CD spectroscopy alone (30), the so-called B-topology has an apparently unique CD signature comprised of a negative band located at wavelengths longer than 220 nm and a positive band at about 200 nm (14). This is precisely what was observed for the differential spectra of indolicidin-L upon interaction with membrane-like environments (Figure 2B). Type II $\beta$-turns, predicted to exhibit B-topology, generally require residue $i + 2$ to be Gly, which is absent from indolicidin. The differential spectra of Figure 2B also resemble the CD appearance of Boc-Tyr-Pro-Phe-Leu-OH reported by Hollosi et al. (30). The latter peptide contains a motif (two aromatic residues separated by proline) shown to favor a type VI $\beta$-turn (8) which differs from type II by cis-Pro isomerization.

The temperature dependence of indolicidin-L (Figure 3A) and indolicidin-F (Figure 3C) in SDS micelles, exhibiting a negative band at 220 nm upon heating, is somewhat puzzling. Similar temperature-dependent transitions reported for unfolded peptides have been attributed to melting of the residual polyproline II conformation (31, 32). However, we did not find indications of such a band upon heating poly-L-proline in either aqueous or membrane-emulating environments (Figure 5). It is possible that this band is inherent to the CD spectrum of unfolded peptides at higher temperatures. Indeed, an increase in negative molar ellipticity around 220 nm has been observed upon heating of various oligopeptides and proteins in unordered states [Dr. S. Yu. Venyaminov, personal communication; see also (33)]. Regardless of the interpretation of the nature and CD appearance of the unfolded state, it is clear that polyproline helices do not contribute to the conformation of membrane-bound indolicidin-L.

Direct NMR measurements (8) and molecular dynamics simulations (12) of small peptides in water demonstrate clearly that proline (Pro) residues with neighboring aromatic residues (Ar) form type VI turns because of the stacking of the aromatics against the Pro ring. Indolicidin contains two Ar-Pro-Ar sequences (Trp$^6$-Pro$^7$-Trp$^8$ and Trp$^9$-Pro$^10$-Trp$^{11}$) and in addition a Leu$^2$-Pro$^3$-Trp$^4$ sequence. All three of these regions of the peptide can reasonably be expected to form turns. Furthermore, the two neighboring Ar-Pro-Ar regions can be expected to bring their tryptophan residues into proximity and thereby permit exciton coupling between the tryptophans. A rather strong coulplet is observed in the CD experiment for indolicidin-W in SDS micelles (Figure 3B) and a couplet of about half that strength for indolicidin-W in membranes (3). This is consistent with the possibility that in the membranes only one out of two Ar-Pro-Ar sequences has the stacked conformation while in the micelles both such sequences are stacked. Such stacking is expected to stabilize the structure of indolicidin-W relative to other analogues, which is consistent with the temperature invariance of the
wild-type CD spectrum in the region 195–200 nm (Figure 3B).

To visualize the appearance of indolicidin in such a membrane-bound conformation, we created molecular models using $\Phi, \Psi$ angles appropriate for type V1a turns (12). One such model with Trp$^b$-Pro$^e$-Trp$^b$ in a turn conformation is shown in Figure 6. It demonstrates that Trp residues can indeed be packed around a cis-Pro stabilizing the turn. According to the theoretical prediction of Grishina and Woody (17), the resulting nearly parallel orientation of the aromatic rings and the distance of 7 Å separating their midplanes are entirely consistent with the strength of the observed exciton coulplet. Interestingly, the conformation of indolicidin shown in Figure 7 places the tryptophans and charged residues on opposite surfaces, suggestive of an amphipathic conformation that could be an important aspect of its high membrane activity. Our results and the general tendency of Ar-Pro interactions to stabilize turns strongly suggest that turns are the most characteristic conformational feature of indolicidin, rather than the polyproline helix. Because tryptophan residues have an exceptionally strong preference for membrane interfaces (10, 34, 35), the partitioning of indolicidin-W into membranes should favor maximum contact of the tryptophans with the interface. We speculate that these contacts stabilize Ar-Pro-Ar turns in the interface that are already favorable in the aqueous phase, a process that we define here as “conformation selectivity”.

The high cost of partitioning of peptide bonds into the membrane interface is believed to be the primary driving force for secondary structure formation on membranes (10, 20, 21), the idea being that hydrogen-bonded peptide bonds have a higher partition coefficient than free peptide bonds. This process is referred to as partitioning—folding coupling (20, 21). Turn formation by indolicidin may achieve a reduction in the peptide bond partitioning cost by an alternate means, namely, by shielding the peptide bonds by the packing of the bulky aromatic side chains around the proline residue (Figure 7). An additional gain might come from weak hydrogen bonding between the backbone and the aromatic rings (36), especially when an aromatic residue follows a cis-Pro (37).

ACKNOWLEDGMENT

We thank Mr. Dat Tran and Dr. George Osapay for their help with the peptide synthesis and Dr. Sergei Yu. Venyaminov for his comments on the manuscript.

REFERENCES

CD Spectra of Indolicidin Antimicrobial Peptides