Structure-Function Analysis of Tritrypticin, an Antibacterial Peptide of Innate Immune Origin*

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The structural requirements for the antibacterial activity of a pseudosymmetric 13-residue peptide, tritrypticin, were analyzed by combining pattern recognition in protein structures, the structure-activity knowledge-base, and circular dichroism. The structure-activity analysis, based on various deletion analogs, led to the identification of two minimal functional peptides, which by themselves exhibit adequate antibacterial activity against *Escherichia coli* and *Salmonella typhimurium*. The common features between these two peptides are that they both share an aromatic-proline-aromatic (ArProAr) sequence motif, and their sequences are retro with respect to one another. The pattern searches in protein structure data base using the ArProAr motif led to the identification of two distinct conformational clusters, namely polyproline type II and β-turn, which correspond to the observed solution structures of the two minimal functional analogs. The role of different residues in structure and function of tritrypticin was delineated by analyzing antibacterial activity and circular dichroism spectra of various designed analogs. Three main results arise from this study. First, the ArProAr sequence motif in proteins has definitive conformational features associated with it. Second, the two minimal bioactive domains of tritrypticin have entirely different structures while having equivalent activities. Third, tritrypticin has a β-turn conformation in solution, but the functionally relevant conformation of this gene-encoded peptide antibiotic may be an extended polyproline type II.

The survival of all living organisms necessitates a rapid and effective host defense against invading pathogens. The higher organisms that have arrived much later in a world inhabited by prokaryotes developed many host defense mechanisms, including gene-encoded antimicrobial peptides to face the challenge of these pathogens (1, 2). The antimicrobial peptides serve as one of the first line of defense against pathogens in vertebrates, they represent the major component of the immune response in invertebrates (3, 4). They are generally localized at specific sites that are exposed to microbial invasion. A class of antibacterial peptides called cathelicidins, which are synthesized as larger precursor molecules in bone marrow, are thus active in polymorphonuclear leukocytes (5). Several members of cathelicidin family have been characterized and include CAP18 from rabbit granulocyte (6); p15 from rabbit polymorphonuclear leukocyte (7); bac5, indolicidin, and cyclic dodecapeptide from bovine neutrophils (8–10); and C12 from porcine bone marrow (11). The shared N-terminal domain in all these precursor molecules is homologous to cathelin, a known host defense molecule of innate immune response (11). A part of the C-terminal domain varying in length from 13 to 30 residues and having no homology among different members of cathelicidins has antimicrobial activity. Tritrypticin is one such 13-residue tryptophan-rich bactericidal peptide derived from C12 (12).

The inherent genetic plasticity on one hand and the ability to adapt to challenging environments on the other have led to the development of antibiotic resistance by many microorganisms (2). The gene-encoded antimicrobial peptides show in vitro activities against microorganisms resistant to conventional antibiotics (2, 13) and could provide impressive design templates for developing potent anti-infectious agents. The structure and mechanism of action of tritrypticin were, therefore, undertaken by a knowledge-based approach involving analysis of conformational patterns in homologous sequences under the experimentally derived structural constraints (14). Here we report the functionally relevant structural features of tritrypticin and shed light on the possible mechanisms associated with its activity.

EXPERIMENTAL PROCEDURES

Materials—4-Hydroxymethyl phenoxymethyl polystyrene resin, solvents, and reagents used for synthesis were supplied by Applied Biosystems Inc. Fmoc1 amino acid derivatives were procured from Nova-biochem and Bachem Feinchemikalien AG. (Bubendorf, Switzerland). Trifluoroacetic acid, 1,2-ethanedithiol, and thioanisole for cleavage were procured from Sigma. Phenol crystals (analytical reagent) and diethyl ether (analytical reagent) were purchased from S. D. Fine Chem. Ltd. (Boisar, India). High performance liquid chromatography grade acetonitrile was obtained from Merck.

The Gram-negative bacterial strains *Salmonella typhimurium* 3261 PNP2 Gro A mutant and *Escherichia coli* BL21(ES) were used for radial diffusion assay. Agarose I (Biotechnology grade) was obtained from Amresco, and tryptic soy broth (TSB) was from Himedia Laboratories Pvt. Ltd. (Mumbai, India). Tween 20 was purchased from Aldrich. Sterilized round Petri plates were purchased from Tarsons (Calcutta, India).

Peptide Synthesis, Purification, and Characterization—Tritrypticin and its analogs were synthesized by solid phase method using an automated peptide synthesizer Model 431A (Applied Biosystems Inc.) employing standard Fmoc methodology. The peptides were cleaved from the resin by treatment with trifluoroacetic acid/thioanisole/phenol/water/1,2-ethanedithiol in ratio as recommended by Applied Biosystems Inc. The crude peptides were purified using C-18 column (Deltapak-100Å, 15 μm spherical, 19 × 300 mm, Waters), and peptide purity was verified using C-18 analytical column (Deltapack-300Å, 15 μm, spherical, 7.8 × 300 mm, Waters). Elution of the peptides was accomplished with a linear gradient from 15 to 80% acetonitrile containing 0.1% trifluoroacetic acid.

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1 The abbreviations used are: Fmoc, N-(9-fluorenylmethoxy carbonyl); TSB, tryptic soy broth; PDB, protein data bank; ArProAr, aromatic-proline-aromatic; PP II, polyproline type II.

2 This paper is available on line at http://www.jbc.org
taining 0.1% trifluoroacetic acid over 30 min. Characterization was performed by molecular mass determination using single Quadruple mass analyzer (Fisons Instruments, Altrincham, UK).

**Antibacterial Assay**—The radial diffusion assay was performed using double-layered agarose as described by Lehrer et al. (15) with slight modification. Bacteria were grown overnight for 18 h at 37 °C in 10 ml of full strength (3% w/v) TSB; 10 μl of this culture was inoculated into 10 ml of fresh TSB and incubated for an additional 3 h at 37 °C to obtain midlogarithmic phase organisms. About 1 × 10⁶ cells were then mixed with 1% agarose in 10 ml sodium phosphate buffer, pH 7.4, containing 0.02% Tween 20 and 0.03% TSB. The mixture was poured into round Petri plates after rapidly dispersing, and a 5-μl peptide sample was placed in each well made in the agarose and then incubated at 37 °C for 3 h. The overlay agar containing 1% agarose in 10 ml sodium phosphate buffer, pH 7.4, and 3% TSB was then poured over it and further incubated at 37 °C for 18–24 h. The diameter of the clear zone surrounding the well was measured for the quantitation of inhibitory activities.

**Circular Dichroism**—The circular dichroism (CD) experiments were carried out on a JASCO 710 spectropolarimeter with 1.0 nm bandwidth at 0.1-nm resolution and 1 s response time using a 10-mm path-length cell. Typically, 20 scans at a speed of 200 nm/min were accumulated at 10 °C and averaged. The peptide concentrations used were 10 μM in water. Results were expressed as mean residue molar ellipticity in deg cm²/dmol.

**Computer Modeling**—BLAST program (16) on Internet was used for sequence searches in the Brookhaven protein data bank (PDB) (17). The BIOSYM software INSIGHTII (Biosym Technologies) was used on IN-DIGO² workstation (Silicon Graphics) for model building, analysis, and display of structural data. Template-based peptide modeling was carried out using the HOMOLOGY module based on the coordinates of individual homologous sequences in the corresponding PDB files. The models were refined in AMBER force field (18) using energy minimization. Distance-dependent dielectric constant was used, and no cross-term energies were included.

**RESULTS**

**Identification of the Smallest Active Analog(s) of Tritrypticin**—The antibacterial activity of tritrypticin (SN13) against *S. typhimurium* and *E. coli*, determined by radial diffusion assay, is shown in Fig. 1. The dose-dependent increase in the antibacterial activity of SN13 was evident in both the cases, although it is slightly more active against *E. coli* than *S. typhimurium*. These curves were used as reference for all the subsequent experiments designed to compare activities of the tritrypticin analogs. Activities of the analogs were assayed at 5 and 50 nmol as both these quantities fall into the linear region of the dose-dependent activity curve. It was observed that the comparative activities of the analogs inferred on the basis of inhibition zone area at 5 and 50 nmol are consistent with each other; therefore, data corresponding only to 5 nmol are given in subsequent comparisons of the activities of tritrypticin analogs.

The shortest active fragment of SN13 was identified by synthesizing various deletion analogs and subjecting them to the antibacterial activity assay. Table I shows different analogs identified by their sequences and an internally consistent code defining each of these peptides. Activity of the peptide is given as the inhibition zone area at the peptide dose of 5 nmol as well as the relative activity (%) with reference to SN13. Behavior of various deletion analogs of tritrypticin appeared similar in the two bacterial strains. As shown in Table I, an analog with deletion of Val-1 (SN12) had activity comparable with that of SN13. Subsequent N-terminal deletions showed progressive decrease in the activity except in case of SN10, which seemed to show drastic reduction in activity to about 22% for *S. typhimurium* and 30% for *E. coli*. Further deletion of another residue (Phe-4) led to regaining of the activity (74% for *S. typhimurium* and 94% for *E. coli*) in case of SN9. SN8 becomes the smallest active fragment of tritrypticin if those analogs with activity more than 50% of the native tritrypticin were defined as active.

The sequence of SN13 is somewhat symmetric, and deletion of Val-1 does not affect its activity. Therefore, it was expected that the deletion of Val-1 and Leu-11 in SN13, which makes it a perfectly symmetric peptide (SYM11) in terms of amino acid sequence, could also be active. It turned out that SYM11 was more active than the native peptide (Table I). Correspondingly, CT7, an analog of SN8 with deletion of Leu-6 was also showing comparatively more activity. The corresponding inverse equivalent N-terminal peptide (NT7) also showed high antibacterial activity (Table I). Thus, NT7 and CT7 are the minimal

![Fig. 1. Dose-dependent activity curve of tritrypticin showing antibacterial activity against *S. typhimurium* and *E. coli* expressed in terms of inhibition zone area in the radial diffusion assay.

| Peptide code | Sequence | Area in mm² (S.E.) at 5 nmol | % inhibition (S.E.) at 5 nmol |
|--------------|----------|-----------------------------|-----------------------------|
| S. typhimurium | E. coli | S. typhimurium | E. coli |
| SN13 | VRRFPWWPFLLR | 106.2 (12.4) | 143.9 (5.3) | 100.0 (11.6) | 100.0 (3.7) |
| SN12 | RRRFPWWPFLLR | 109.1 (8.0) | 144.4 (5.6) | 102.7 (7.5) | 100.3 (3.9) |
| SN11 | RRFPWWPFLLR | 85.3 (9.0) | 137.1 (1.8) | 80.3 (8.5) | 95.3 (1.3) |
| SN10 | FPWWPFLLR | 23.5 (1.5) | 42.8 (1.0) | 22.1 (1.4) | 29.7 (1.0) |
| SN9 | PWWPFLLR | 79.0 (4.4) | 135.4 (4.7) | 74.4 (4.3) | 94.1 (3.3) |
| SN8 | WWPFLLR | 60.1 (6.5) | 69.0 (3.6) | 56.6 (6.1) | 47.9 (2.5) |
| SN7 | WPFLLR | 16.0 (5.4) | 17.5 (1.7) | 15.1 (5.1) | 12.2 (1.2) |
| SN6 | WFLRR | 0.0 (0.0) | 5.8 (2.6) | 0.0 (0.0) | 4.0 (1.8) |
| SYM11 | RRFPWWPFLLR | 144.4 (6.4) | 177.4 (10.2) | 135.9 (6.0) | 123.3 (7.1) |
| CT7 | WWPFLLR | 78.4 (4.9) | 111.8 (3.1) | 73.8 (4.6) | 77.7 (2.2) |
| NT7 | RRFPWWN | 88.0 (9.7) | 126.9 (8.8) | 82.9 (9.1) | 88.2 (6.1) |
bioactive analogs of tritrypticin.

Search for Conformational Patterns Associated with the Minimal Bioactive Analogs—The sequences of NT7 and CT7 are related, considering that they essentially represent two halves of a symmetric larger peptide. The design of their sequences is such that the two peptides are mirror images of each other. They both are made up of a central tripeptide sequence motif, aromatic-proline-aromatic (ArProAr), with two cationic residues on one side and two tryptophans on the other. The conformational preferences of the ArProAr sequence motif, common among these two almost equally active seven-residue peptides, were analyzed in PDB using \( \text{ArProAr} \) as the search sequence, where \( X \) is any amino acid. The search using BLAST led to the identification of 45 unique 7-residue sequences incorporating this motif.

The least square superimpositions of these seven-residue segments were superimposed using the coordinates from the corresponding protein structures in PDB for each of the two groups. Group I consisted of 15 different segments having backbone torsion angles approximately corresponding to polyproline type II (PP II) conformation. The central five residues of these structures could be superimposed such that the corresponding \( C^\alpha \) atoms overlap completely (Fig. 2A). On the other hand, group II consisted of 13 segments exhibiting type III \( \beta \)-turn. The residues 3–6 could be superimposed in all these entries such that the \( C^\alpha \) atoms overlap in this case as well (Fig. 2B). The remaining 17 sequences were widely distributed such that they could not be classified into any major conformational cluster.

The backbone torsion angles for group I and II were analyzed by help of the Ramachandran plot (Fig. 3). The \( \phi \) and \( \varphi \) values of overlapping residues of the 7-residue segments in group II (Fig. 3A) and group I (Fig. 3B) were indicated on the Ramachandran plot. The residues 3, 4, and 5 in group I showed
Fig. 3. The conformational clusters corresponding to ArProAr motif in the protein data bank identified from the Ramachandran ($\phi$$\psi$) plot for $\beta$ turn (A) and PP II (B) conformations. The residues 4 and 5 in the case of $\beta$ turn and 3, 4, and 5 in case of PP II have been plotted superimposed with the allowed regions of the Ramachandran plot.
clustering around the average $\phi$ and $\varphi$ values of $-75^\circ$, $+140^\circ$ representing PP II conformation, and the residues 4 and 5 in group II showed clustering around the average $\phi$ and $\varphi$ values of $-60^\circ$, $-30^\circ$ representing type III $\beta$-turn. Because the $\beta$-turn is stabilized by an hydrogen bond, the spread in the backbone torsion angles in this case is less than in the case of PP II, which is an extended conformation. The PP II conformation also extends to the next residue on either side in majority of the cases among this group.

The structural models for CT7 and NT7 were built using the template coordinates of segments in group I and II, respectively. The van der Waals surface drawings of these models, color-coded to indicate the hydropathy nature of the residues, are shown in Fig. 4, A and B, respectively. There was a distinct clustering of the aromatic residues and the cationic residues in both these groups. However, the clustering was more prominent in group I, which forms PP II structure, compared with group II, which forms $\beta$-turn structure. Also, these clusters are spaced differently in groups I and II. The average distance between the center of gravity of the cationic cluster and that of the hydrophobic cluster was about 10 Å for $\beta$-turn structures and about 16 Å for PP II structures. In case of the other structures, which did not fall into either of the common patterns, such an obvious segregation could not be easily defined.

Structure-Function Analysis of Minimal Bioactive Analogs—The solution structures of the minimal bioactive analogs were investigated. The CD profiles of NT7 and CT7 in aqueous medium are shown in Fig. 5. The profile in the 250–190-nm range suggested that the two analogs each have a definitive, although very different structure in solution. CT7 showed a mean residue molar ellipticity minima at 206 nm, characteristic of the PP II conformation. NT7, which has mirroring sequence with respect to CT7, showed a maximum at 212 nm that corresponds to $\beta$-turn conformation. Both these peptides exhibited a prominent CD signal at around 225 nm, which arises primarily from the interactions of the aromatic tryptophan residues (19). This signal showed a negative peak in the case of NT7 but an opposite, positive and equal peak at the same wavelength for CT7. The antibacterial activity of NT7 as well as CT7 decreased in the presence of Mg$^{2+}$ (data not shown) as in case of tritrypticin (12), an observation suggested to represent functional measure of the cationic peptide antibiotics (2).

Structure-Function Analysis of Tritrypticin—Antibacterial activity of native tritrypticin was compared with that of different deletion analogs of SN13 to delineate the functional role of different N- and C-terminal residues (Table I and Fig. 6A). As described earlier, deletion of a pair of N-terminal cationic residues (SN10) led to drastic loss in activity. Similarly, NT9, the peptide arising from deletion of the C-terminal cationic residues (RRFPWWWPF) also led to significant loss in activity. The deletion of Phe-1 in SN10 (leading to SN9) resulted in enhancement of activity compared with that of SN10. Substi-
tution of all the arginines to lysines in the symmetric peptide SYM11 led to an analog (SYM11KK) with activity comparable with that of SYM11 and about 140% that of native tritrypticin.

The CD profile of SN13 is shown in Fig. 6B. The positive mean residue molar ellipticity at 212 nm suggests a characteristic β-turn conformation. In addition, there was distinct negative mean residue molar ellipticity at 225 nm arising from the interaction of the tryptophan side chain with the backbone involving nearest-neighbor residues. Thus, the predominant structural feature revealed by CD is a β-turn, although there was a small signal at 196 nm corresponding to an extended conformation. Fig. 6B also shows the comparison of CD profiles of the deletion analogs SN10, SN9, SN8, and NT9 with that of native SN13. SN10 and NT9, which had negligible antibacterial activity, exhibited an enhanced β-turn signal with corresponding loss in the PP II component. However, SN9, which had regained activity compared with SN10 with deletion of N-terminal Phe, appears to exist as a combination/mixture of β-turn and PP II conformations. A complete transition of conformation from β-turn in the case of native peptide to PP II in the case of SN8 was observed with further deletion of a proline. The CD signal at 225 nm also changed sign in this case. The symmetric analog, SYM11, has enhanced β-turn character compared with SN13. Similarly, SYM11KK also exhibited CD profile similar to that of SYM11 (Fig. 6C). Both these peptides did not exhibit any minima in the region of 196–206 nm, perhaps indicating that they do not have any fraction of the structure in either extended or PP II conformation.

The SN13 analogs, in which the tryptophan residues were substituted by either an aromatic residue (tyrosine) or a non-aromatic residue (serine), were analyzed to characterize the role of tryptophans in antibacterial activity and structural integrity. All the three tyrosine substituted analogs showed about 25% enhancement in the activity (Fig. 7A). The analogs with nonaromatic substitution at any of the three positions were marginally more active than those substituted by tyrosine. The comparisons of the CD profiles of tyrosine- and serine-substituted analogs with that of SN13 are shown in Fig. 7, B and C, respectively. The substitution of Trp-8 → Tyr showed a decrease in the β-turn signal at 212 nm and appearance of a

![Fig. 5](image)

**Fig. 5.** The CD profiles of the N- and the C-terminal 7-mer peptides, NT7 (RRFPWWW) and CT7 (WWWPFRR), respectively, indicate that the structural behavior of these peptides is consistent with the structural features arising from the pattern search.

![Fig. 6](image)

**Fig. 6.** Antibacterial activity data of various deletion analogs against *S. typhimurium* (A), CD spectra of the N-terminal deletion analogs (B), and the symmetric analogs (C) compared with tritrypticin, suggesting that the terminal cationic residues do not significantly affect the structure but are critical for activity.
small signal at 206 nm. The 225-nm signal, however, did not change significantly. The substitution of Trp-7 → Tyr led to more pronounced changes. The β-turn signal at 212 nm completely disappeared, and the signal at 206 nm corresponding to PP II conformation became very prominent. The 225-nm signal corresponding to the interaction between tryptophan side chains and the backbone of aromatic residues in this case is almost negligible. The Trp-6 → Tyr substitution led to the conversion of conformation from β-turn to PP II in every respect. There is a significant negative mean residue molar ellipticity corresponding to PP II, a pronounced positive peak at 225 nm, and absolutely no signal corresponding to the β-turn. Similar but somewhat more prominent changes were observed in the analogs with serine substitutions for each of the tryptophan residues.

**DISCUSSION**

Tritrypticin belongs to the class of cationic antibacterial peptides. Generally, the cationic peptides have two distinguishing features. These molecules are amphipathic, and they carry a net positive charge of at least +2 (2). The cationic peptide antibiotics are potent candidates for countering antibiotic resistance developed by the microbes against established antibiotics. Although there are inherent difficulties in exploiting peptidyl molecules as drug candidates (20), they provide ideal templates for peptidomimetic design with enhanced half-life and potency while maintaining structure and specificity. The cationic antibacterial peptides show pronounced structural heterogeneity. The three-dimensional structures of at least one member of three different families of peptidyl antibiotics have been determined (21–25). As the functional specificity and mechanism of killing are obviously dependent on the three-dimensional structure and chemical nature of the peptide, diverse families of cationic antibacterial peptides are unlikely to adopt a common mechanism of action. The structural insights gained in the functional context could make tritrypticin among the most suitable candidates for peptidomimetic design.

Tritrypticin is a pseudosymmetric molecule. Although it was suggested that the 13-residue tritrypticin is a processed antibiotic (12), alignment of the precursor sequence with other cathelicidin precursors implies that Val-1 in this sequence should correspond to the elastase cleavage site (11). Therefore, Val-1 in tritrypticin probably has no functional role. The comparison of SN12 and SN13 activities confirmed this interpretation. A symmetric analog (SYM11) also shows enhanced antibacterial activity. Besides, NT7 and CT7 are both active and may correspond to two independent minimal functional domains. This would imply that the symmetric composite peptide SYM11 or the native SN13 are naturally designed to enhance activity by some sort of duplication. NT7 and CT7 are not entirely unrelated. Their sequences are retro with respect to each other. Another common feature of these two minimal bioactive peptides is the ArProAr motif incorporated within their sequences. A couple of other peptides having similar sequence features have also been shown to possess definitive structural folds (26, 27). Considering that both NT7 and CT7 are equally active, this common motif was critical in deriving the structural and functional features associated with their antibacterial activities.

Pattern recognition in the protein structure data base led to the identification of two distinct conformational motifs, namely PP II and β-turn, for the ArProAr sequence motif. It can be inferred that the ArProAr sequence motif has an inherent
structural preference for one of these two conformations. Although linear peptides of this size are normally observed to have multiple conformational populations in solution, the present case appears to be different. The structural motifs associated with the ArProAr sequence motif in PDB appear to have some additional sequence constraints imposed by the nature of residues downstream of the ArProAr sequence. The consensus in one of the two residues on the right side of ArProAr motif is a charged residue for the group I, which adopts PP II conformation. Similarly, consensus among the two residues at this position is hydrophobic in group II, which adopts b-turn conformation. Identification of the conformational patterns associated with specific sequence signatures is relevant in the context of protein design rules. The architectural design of proteins is such that a finite number of structural modules are used again and again in different contexts and combinations (28, 29). Infinitely diverse overall topologies associated with equally diverse functions seem to have emerged from this clever design. Analyses of many such independent structural modules reveal that it is possible to define sequence signature of a motif by identifying certain invariant residues, which occur at equivalent positions in a consensus manner (30–33). The two structural folds associated with their respective sequence signatures are the important addition to this library of structural modules.

Two structural motifs derived from the patterns in protein structures are consistent with the solution conformations of the two minimal bioactive analogs. NT7, which exhibits b-turn conformation, has two hydrophobic residues following the ArProAr motif, and CT7, which shows PP II conformation, has two charged residues following the ArProAr motif, similar to the consensus feature observed in the protein data base. Both the conformations show clear amphipathic nature with the segregation of cationic residues and aromatic residues. However, the distance between these clusters is more in the case of the PP II conformation compared with the b-turn conformation. This may have direct implications to achieving complementarity vis à vis the receptor on the membrane. The ArProAr motif with similar structural preferences has been characterized in peptides among certain other contexts as well (26, 27).

One of the characteristic features of the CD profiles of tritrypticin analogs is the dichroic signal at about 225 nm, which is expected to arise because of the conformational environment of the tryptophan residue. The asymmetric indole derivative of the tryptophan side chain could lead to either positive or negative circular dichroic rotation depending on the backbone conformation in the immediate neighboring residues (19). All the analogs discussed in the present study have multiple tryptophan residues. It is evident that in some of them the mean residue molar ellipticity is positive, and in some others it is negative. However, there is a direct correlation of the sign of ellipticity at 225 nm with secondary structural characteristics. All the peptides with negative molar ellipticity at 225 nm have a distinct positive signal at 212 nm corresponding to b-turn. Similarly those peptides that exhibit positive mean residue molar ellipticity at 225 nm exhibit PP II structure as indicated by a negative signal at 206 nm. Thus, the characteristic circular dichroic signal arising from the aromatic side chain-peptide backbone interactions is also linked with the two distinct conformational states associated with this motif.

Tritrypticin has a definitive structure in solution. There is a clear b-turn signal, but also, in addition, there is a small minimum corresponding to an extended structure in the CD profile of the native peptide. The minima corresponding to the extended structure began to shift toward PP II and appeared prominently as the residues from the N-terminal were sequentially deleted. The conformational features of the individual minimal functional domains of SN13, namely NT7 and CT7, have direct correlation with the conformation of tritrypticin. Obviously, the N-terminal domain of tritrypticin, which essentially corresponds to NT7, can be suggested to have a b-turn-fold, and the C-terminal domain, which primarily constitutes CT7, can be suggested to have PP II or extended conformation in the molecule. The three tryptophan residues, apparently important for structure as well as activity, are actually shared by both the domains. It is also evident that the charge and not the nature of the side chain is important for activity in the case of the terminal cationic residues. SN10 and NT9 may have some shielding effect of the terminal Phe residue while binding to the relevant membrane receptor, resulting in abnormally low activity. It is clear that the amphipathic nature of the peptide alone is not adequate for its activity, as the smaller amphipathic analogs were not active.

Tritrypticin apparently undergoes a conformational transition while approaching the membrane receptor. The majority of the small bioactive peptides, which are not constrained through an intramolecular disulfide bridge, undergo a transition from an unstructured to a structured active form in the vicinity of the receptor (14). A disorder-order transition is involved in the activation of such peptides in most cases where the peptide does not indicate any preferred conformation. Tritrypticin is unique in that it adopts a well defined type III b-turn conformation in solution. The minimal functional analogs of tritrypticin, NT7 and CT7, show b-turn conformation and PP II conformation, respectively, and both are equally active. Many different single substitution analogs of tritrypticin show enhanced antibacterial activity accompanied by a change in conformation, from b-turn to PP II. It can therefore be inferred that the functional activation of tritrypticin may involve a transition from the solution conformation constituting a b-turn to the bioactive conformation, which is predominantly PP II type.

The precise mechanism of bacterial killing by tritrypticin is not known. A diverse array of mechanisms by which other peptidyl antibiotics attack the bacterial cell have been proposed. Many cationic antibiotic peptides are suggested to be membrane-active and assemble forming channels (34–35). Alternatively, certain peptides cluster at the membrane surface and cause a cooperative permeabilization by the carpet effect (36). On the other hand, apidaecins function through a receptor-activated nonpore-forming mechanism involving steerspecificity (37). The bactenecins are suggested to cause loss of macromolecular synthesis ability (38). The nonlytic PR-39 kills bacteria by interrupting both DNA and protein synthesis (39). The DNA binding property of tachyplesin I has also been implicated in the antibiotic activity (40). The observed differences in the mechanism for bacterial killing appear to be consistent with the structural diversity among these molecules. However, the initial event common to all the cationic peptides is the binding of positively charged residues to the negatively charged molecules exposed at the target cell surface. The peptide antibiotics show differential activities against different bacterial strains, which may be related to the differences in the composition of the cell surface molecules. The tritrypticin and its minimal functional domains exhibit activity against at least two different Gram-negative bacteria. It does not necessarily imply that it would similarly work against any bacterial strain. The amphipathic structural design may be relevant for the specificity of tritrypticin. It may be achieved by clustering of the hydrophobic residues and cationic residues on its either side, appropriately distanced as in any PP II structure, such that it can match a complementary site on the receptor. Both
the hydrophobic clustering and the electrostatic interactions accompanied by the relative flexibility in the peptide molecule would provide certain leeway within this specificity.

In summary, tritrypticin has predominantly \( \beta \)-turn conformation in its N-terminal region and is designed by duplication to have enhanced activity involving two independent functional domains. More than half as active as tritrypticin, these domains are functionally equivalent but structurally very different from each other. As an initial event in bacterial killing, tritrypticin apparently undergoes functional activation through a conformational transition from \( \beta \)-turn to \( \alpha \)-helix while specifically binding to the negatively charged receptor exposed at the target bacterial membrane. The specificity of tritrypticin binding to the membrane surface may be achieved by the appropriate juxtaposition of the hydrophobic residues and the cationic residues so as to match a complementary site on the receptor.

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