Chitin-binding Proteins in Invertebrates and Plants Comprise a Common Chitin-binding Structural Motif* 

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Tachycitin, a 73-residue polypeptide having antimicrobial activity, is present in the hemocyte of the horseshoe crab (Tachypleus tridentatus). The first three-dimensional structure of invertebrate chitin-binding protein was determined for tachycitin using two-dimensional nuclear magnetic resonance spectroscopy. The measurements indicate that the structure of tachycitin is largely divided into N- and C-terminal domains; the former comprises a three-stranded β-sheet and the latter a two-stranded β-sheet following a short helical turn. The latter structural motif shares a significant tertiary structural similarity with the chitin-binding domain of plant chitin-binding protein. This result is thought to provide faithful experimental evidence to the recent hypothesis that chitin-binding proteins of invertebrates and plants are correlated by a convergent evolution process. 

An invertebrate chitin-binding protein named tachycitin is recently found to be a member of the primordial elements of innate immune defense against bacterial and fungal infections (1–5). The antimicrobial activity is initially identified for chitin-binding proteins extracted from plants (6–7), which commonly comprise single or multiple copies of the chitin-binding domain. The plant chitin-binding domain is mostly composed of 30–43 residues including eight cysteines, three aromatic residues, and glycines and is frequently referred to as a hevein domain (8). It has been well demonstrated that this domain is indispensable for the antimicrobial activity and exhibits a significant conservation in primary sequence (>40%) and in three-dimensional (3D) structural (9–12). Although this advanced knowledge has been provided for the plant chitin-binding proteins, less is known for the invertebrate chitin-binding proteins including tachycitin (1, 13–18). Kawabata et al. (1) identified that tachycitin is a 73-residue chitin-binding protein having antimicrobial activity. They also revealed that tachycitin consists of five intramolecular disulfide bridges; the connected Cys pairs are 6–33, 12–30, 24–61, 25–68, and 40–53. For invertebrates, the chitin-binding domain was assumed to comprise about 65 residues (17) involving a high percentage of cysteine and aromatic residues in a similar manner to the plant chitin-binding domain. On the basis of such similarity between plant and invertebrate chitin-binding proteins, Shen and Jacobs-Lorena (17) proposed a hypothesis that they are correlated by a rare evolutionary process, convergent evolution, i.e., proteins from different origins develop to construct the same active site structure to acquire the same function. However, complete lack of 3D-structural information of invertebrate chitin-binding protein obscures the evolutionary relationship between invertebrate and plant chitin-binding proteins. The present study determines the solution structure of tachycitin using NMR spectroscopy, which provides the first 3D structural information of invertebrate chitin-binding protein. 

EXPERIMENTAL PROCEDURES 

An invertebrate chitin-binding protein, tachycitin, was isolated from hemocyte debris of horseshoe crab (Tachypleus tridentatus) as described previously (1) and used without further purification. The NMR samples were prepared by dissolving tachycitin in either 0.3 ml of D2O or H2O containing 10% D2O to give a final concentration of 1–2 mM, whose pH values were adjusted to be 4.0–5.5 by addition of DCl and/or NaOD. The NMR experiments were performed on JEOL JNM-Alpha 500 and 600 spectrometers operating at temperatures of 15, 20, 30, and 40 °C. The two-dimensional experiments, DQF-COSY, TOCSY (mixing time 75, 85 ms), and NOESY (mixing time 75, 250 ms), were acquired with low-power (20 Hz) presaturation on the water. The temperature coefficient (∆δ/∆T, ppb K–1) was estimated from the temperature dependence (15–40 °C) of the chemical shift of the HN resonances. The chemical shifts were referenced to the internal standard, TSP (0.00 ppm). Inter proton distance restraints were derived from NOE cross-peaks in the NOESY spectra (mixing time = 75 ms), calibrated the peak intensities with known distances (2.2 Å for H–H; 1.75 Å for H–D), and were used as inputs for 3D structural calculations of tachycitin. The NOEs were classified into strong, medium, and weak, corresponding to three distance restraints with an upper limit of 2.7, 3.5, and 5.0 Å, respectively. The upper distance limit was corrected for methyl and methylene protons that were not assigned stereospecifically. The 35 dihedral φ angle restraints were obtained by measuring (JHN-Hα coupling constants; the φ angle restraint of −60 ± 30° was used for the residues having JHN-Hα coupling constants less than 6 Hz, and that of −120 ± 30° was used for the residues having JHN-Hα coupling constants larger than 8 Hz. Hydrogen bond distance restraints were applied between nitrogen and oxygen atoms (2.3–3.5 Å) and HN and oxygen atoms (1.5–2.5 Å) for regular secondary structures. The hydrogen bonding was assumed for the residues 18, 27–29, 31, 34, 35, 45, 47, 52, 54, and 59, which show low temperature coefficients

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The atomic coordinates and structure restraints (code 1DQC) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/) 

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TABLE I

NMR-derived restraints and structural statistics for the 25 calculated structures of tachycitin

| No. of distance restraints | Total | Short | Medium (2 ≤ |j| − |j′| ≤ 4) | Long (|j| − |j′| ≥ 5) | Hydrogen bond | Disordered restraints (d) | Restraint violation | RMSD (Å) |
|---------------------------|-------|-------|-----------------|-------------------|----------------|----------------|------------------------|-------------|----------|
|                           | 1035  | 488   | 326                      | 195              | 96           | 35            | 0                      | 0          |

- Well defined region
- Backbone atoms (heavy atoms)
- β-Sheet regions
- Backbone atoms (heavy atoms)

| Energies (kcal/mol) | F:<sup>total</sup> | F:<sup>bonds</sup> | F:<sup>angles</sup> | F:<sup>impropers</sup> | F:<sup>nondep Waals</sup> (F repel) | F:<sup>NOE</sup> | F:<sup>improper</sup> |
|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                     | 125.93 ± 8.75       | 3.92 ± 0.76         | 93.08 ± 5.01        | 14.95 ± 1.41        | 9.52 ± 2.84         | 4.20 ± 1.35         | 0.26 ± 0.16         |

| NOE distance restraints (Å) | 0.0089 ± 0.0014 |
| Dihedral angle restraints (degrees) | 0.3339 ± 0.1036 |
| RMSD from ideal covalent geometry | 0.001833 ± 0.000180 |
| Bonds (Å) | 0.5424 ± 0.0146 |
| Improper (Å) | 0.3912 ± 0.0182 |

- φ and ψ in core and allowed regions (%) 97.3

(≤4.5 ppm K<sup>−1</sup>) Our previously described disulfide bond formations were also used as the distance restraints (1). All NMR data were processed using NMRPipe (18) and PIPP (19), and the structure calculations were performed using simulated annealing (SA) protocol in X-PLOR 3.851 (20). An extended structure of tachycitin was used as the starting structure for calculation, whose C-terminal end is pathed (1) with an amide group, CONH<sup>2</sup>. The initial set of restraints included only NOE restraints, no dihedral restraints, and no hydrogen bond restraints. From this initial set of restraints, 100 structures were generated using the SA protocol with heating for 30 ps and cooling for 30 ps. Of those 100 structures, 50 structures with lower total energy were selected as starting structures for the next refinement cycles. The refinement cycles were performed using the SA protocol with heating for 30 ps and cooling for 20 ps. The 25 lowest-energy structures presented in this paper were selected from the 50 structures calculated from the final round of refinement. The program PROCHECK (21) reveals that for all residues, 97.3% of the backbone φ and ψ dihedral angles fell into core and allowed regions of the Ramachandran map.

RESULTS AND DISCUSSION

The structural determination was performed (Table I) using NMR-derived 1,070 experimental restraints, on the basis of the whole assignments of the 1H resonances of tachycitin at pH 4.2 and at 30 °C, which were deposited to BioMagResBank with the accession number of 4290. Structure of tachycitin (Fig. 1) appears to comprise a three-stranded β-sheet (β1, β2, and β3; residues 17–19, 26–31, and 34–39) in the N-terminal region and a two-stranded β-sheet (β4 and β5; residues 45–47 and 52–54) following a short helical turn (α1; residues 56–59) in the C-terminal region. Such arrangements of the secondary structures of tachycitin are not similar to those of any other known antimicrobial peptides in invertebrates; for example, the insect defensin family consists of one long loop, one α-helix, and one β-sheet from the N terminus (22). As shown in Fig. 1B, a distorted β-sandwich structure is constructed by the three-stranded and two-stranded β-sheets connected through a bending loop (Cys-40–Leu-44). It appears that this bending loop involves a type III β-turn contributed by the residues Pro-41–Leu-44, for which the formation of a hydrogen bond between Leu-44 H<sup>N</sup> and Pro-41 O<sup>ψ</sup> is evidenced by low temperature coefficient of Leu-44 (3.0 ppm K<sup>−1</sup>). A short segment (residues His-31–Leu-34) flanked between the strands β2 and β3 constructs a β-turn conformation. For this β-turn, molecular motion restraint contributed by a disulfide bond (Cys-6–Cys-33) is suggested by observations of significant line-broadening of the H<sup>N</sup> resonances for Lys-32 and Cys-33. Another segment comprising six residues (Asn-47–Val-52) flanked by strands β4 and β5 adapt a β-hairpin structure. In this β-hairpin, Asn-47 O<sup>ψ</sup> presumably forms a hydrogen bond with the Lys-51 H<sup>N</sup>, which is supported by the extremely low temperature coefficient (2.0 ppm K<sup>−1</sup>) obtained for Lys-51. Overall, the structure of tachycitin is characterized by β-sheets flanking short loops and turns, which is typical for most of the small disulfide-rich polypeptides (23).

It was revealed that tachycitin shares a remarkable local structural similarity with a plant chitin-binding protein named hevein. Comparison between our determined structure of tachycitin (Fig. 2A) and a previously reported structure of hevein (9) (Fig. 2B) clearly shows that an antiparallel β-sheet (colored in blue) and a helical turn (colored in red) are constructed in both proteins in highly similar manners. In addition, formation of a disulfide bridge (between Cys-40 and Cys-53) connecting the middle of β5 and the C terminus of β3 for tachycitin (colored in green, Fig. 2A) is similarly identified in hevein (Fig. 2B). The structural similarity further includes the loop regions, e.g. a hairpin loop structure involved in the anti-parallel β-sheet (colored in orange). It should be noted that the hairpin loop of tachycitin (Asn-47–Val-52) comprises six residues with βαγαββ conformation whereas the corresponding loop of hevein comprises five residues with βαγαββ conformation.

Kawabata et al. (1) reported that the N-terminal 5–28 region of tachycitin shows sequence similarity with the N-terminal 2–21 region of hevein. However, such similarity is not identified by the present study; the secondary structural arrangement, as well as the disulfide-bond patterns, appears to be quite different for the suggested regions.

The structural similarity between segment Cys-40–Gly-60 of tachycitin and segment Cys-12–Ser-32 of hevein, both comprising the antiparallel β-sheet (β4 and β5), was examined by looking at the superimpositions of the segments (Fig. 3). The structural motif shown in Fig. 3 has been found in several plant chitin-binding proteins (10–12) (Fig. 4). For hevein, segment Cys-12–Ser-32 was identified as an essential chitin-binding domain (24). It appears that arrangements of the two structural motifs shown in Fig. 3 are significantly consistent with each other (backbone RMSD = −1.5 Å). The aromatic side-chains groups of Trp-21 and Trp-23 of hevein (Fig. 3) are known to bind specifically to chitin-derived oligosaccharides through hydrophobic interactions (24, 25). This binding is further strengthened by a hydrogen bonding with Ser-19 of hevein (25). As shown in Fig. 3, the residues of Asn-47, Tyr-49, and Val-52 of tachycitin are located at perfectly corresponding positions to the residues of Ser-19, Trp-21, and Trp-23 of hevein. Therefore, one could assume that the region shown in Fig. 3 comprising an antiparallel β-sheet and a helical turn (β4, β5, and α1; Fig. 2A) in the C-domain of tachycitin serves as an essential chitin-binding site, which protrudes the side-chains of the putative functional residues, Asn-47, Tyr-49, and Val-52. Overall, it could be assumed that the N-terminal region comprising β1–β3...
of tachycitin (colored in gray in Fig. 2A) behaves as a stable domain so as to locate the C-terminal domain chitin-binding site proper for its function.

Conservation of the chitin-binding structural motif among the chitin-binding proteins in invertebrates and plants was further examined by alignment tests of the proteins with regard to their amino acid sequences corresponding to Cys-40–Gly-60 of tachycitin (Fig. 4). The 3D structural information has been available for the plant chitin-binding proteins (9–11). The information is now available for only tachycitin among the invertebrate chitin-binding proteins. It appears that the residues of Cys, Pro, and Gly, all of which have significant influence on the structural constructions, are well conserved in the chitin-binding proteins listed in Fig. 4. Conservation of polar and hydrophobic residues is further identified for the putative chitin-binding residues (e.g. Asn-47, Tyr-49, and Val-52 for tachycitin). For all plant chitin-binding proteins, the 21-residues segments listed in Fig. 4 appear to construct a closely similar 3D structure (9–11) to the putative chitin-binding site of tachycitin. Further similarity in primary sequence identified between tachycitin, Ag-chit, Pj-chit1, Ch-chit, Peritrophin-44, and Tn-IM (nomenclatures described in the figure legend) as assumed that these segments of the invertebrate chitin-binding proteins commonly comprise the chitin-binding structural motif as identified in tachycitin. In 1999, Shen and Jacobs-Lorena (17) proposed a hypothesis that chitin-binding proteins in invertebrates and plants are correlated by a rare evolutional process, convergent evolution. Our present structural determination of tachycitin and the 3D structure-based sequence alignment are thought to provide faithful evidences for the proposed idea of the convergent evolution relationship between invertebrate and plant chitin-binding proteins.

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REFERENCES

1. Kawabata, S., Nagayama, R., Hirata, M., Shigenaga, T., Agarwala, K. L., Saito, T., Cho, J., Nakajima, H., Takagi, T., and Iwanaga, S. (1996) J. Biochem. 120, 1253–1260
2. Kawano, K., Yoneya, T., Miyata, T., Yoshikawa, K., Tokunaga, F., Terada, Y., and Iwanaga, S. (1990) J. Biol. Chem. 265, 15365–15367
3. Beisel, H. G., Kawabata, S., Iwanaga, S., Huber, R., and Bode, W. (1999) EMBO J. 18, 2313–2322
4. Hoess, A., Watson, S., Siber, G. R., and Liddington, R. (1993) EMBO J. 12, 3351–3356
5. Iwanaga, S., Kawabata, S., and Muta, T. (1998) J. Biochem. 123, 1–15
6. Broekaert, W. F., Marie, W., Terras, F. R., De Bolle, M. F., Proost, P., Van Damme, J., Dillen, L., Claes, M., Rees, S. B., Vanderleyden, J., and Cammue, B. P. (1992) Biochemistry 31, 4308–4314
7. Koo, J. C., Lee, S. Y., Chun, H. J., Cheong, Y. H., Choi, J. S., Kawabata, S., Miyagi, M., Tsunasawa, S., Ha, K. S., Bae, D. W., Han, C. D., Lee, B. L., and Cho, M. J. (1998) Biochim. Biophys. Acta 1382, 80–90
8. Beintema, J. J. (1994) FEBS Lett. 350, 159–163
9. Andersen, N. H., Cao, B., Rodriguez-Romero, A., and Arreguin, B. (1993) Biochemistry 32, 1407–1422
10. Martino, J. C., Maes, D., Loris, R., Pepermans, H. A., Wyns, L., Willem, R., and Verheyden, P. (1996) J. Mol. Biol. 258, 322–333
11. Wright, C. S. (1996) J. Mol. Biol. 215, 635–651
12. Weaver, J. L., and Prestegard, J. H. (1998) Biochemistry 37, 116–128
13. Elvin, C. M., Vuocolo, T., Pearson, R. D., East, I. J., Riding, G. A., Eisemann, C. H., and Tellam, R. L. (1996) J. Mol. Biol. 271, 8925–8935
14. Shen, Z., and Jacobs-Lorena, M. (1997) J. Biol. Chem. 272, 28895–28900
15. Shen, Z., and Jacobs-Lorena, M. (1998) J. Biol. Chem. 273, 17665–17670
16. Watanabe, T., Kono, M., Aida, K., and Nagasawa, H. (1998) Biochim. Biophys. Acta 1382, 181–185
17. Shen, Z., and Jacobs-Lorena, M. (1999) J. Mol. Biol. 28, 341–347
18. Delaglio, F., Grzesiek, S., Vuister, G. W., Zhu, G., Pfeifer, J., and Bax, A. (1995) J. Biomol. NMR 6, 277–293
19. Garrett, D. S., Powers, R., Gronenborn, A. M., and Clore, G. M. (1991) J. Magn. Reson. 95, 214–220
20. Brunger, A. T. (1992) X-PLOR: A System for X-ray Crystallography and NMR, Version 3.851, Yale University Press, New Haven, CT
21. Laskowski, R. A., MacArthur, M. W., Moss, D. S., and Thornton, J. M. (1993) J. Appl. Crystallogr. 26, 283–290
22. Dimarco, J. L., Bulet, P., Hetru, C., and Hoffmann, J. (1998) Biopolymers 47, 465–477
23. Harrison, P. M., and Sternberg, M. J. (1996) J. Mol. Biol. 264, 603–623
24. Asensio, J. L., Canada, F. J., Bruix, M., Rodriguez-Romero, A., and Jimenez-Barbero, J. (1995) Eur. J. Biochem. 230, 621–633
25. Asensio, J. L., Canada, F. J., Bruix, M., Gonzalez, C., Khair, N., Rodriguez-Romero, A., and Jimenez-Barbero, J. (1998) Glycobiology 8, 569–577
26. Kam, R., Biletier, M., and Wuthrich, K. (1996) J. Mol. Graph. 14, 51–55
27. Watanabe, T., Kono, M., Aida, K., and Nagasawa, H. (1996) Mol. Mar. Biol. Biotechnol. 5, 299–303
28. Krishnan, A., Nair, P. N., and Jones, D. (1994) J. Biol. Chem. 269, 20971–20976
29. Wang, P., and Granados, R. R. (1997) J. Biol. Chem. 272, 16663–16669

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