Fungal Metabolites. XX.\(^1,2\) Effect of Proline Residue on the Structure of Ion-Channel-Forming Peptide, Trichosporin B-Vla

YASUO NAGAOKA,\(^a\) AKIRA IDA,\(^a\) EIICHI TACHIKAWA,\(^b\) and TETSURO FUJITA\(^*a,d\)

Faculty of Pharmaceutical Sciences, Kyoto University,\(^a\) Sakyoku, Kyoto 606-01, Japan and School of Medicine, Iwate Medical University,\(^b\) Morikawa 020, Japan. Received February 27, 1995; accepted April 10, 1995

The secondary structures of an ion-channel-forming isoapetiaol, trichosporin B-Vla, and its Aib\(^14\)-substituted derivative containing no Pro were investigated on the basis of CD and various NMR experiments in methanol. Trichosporin B-Vla has a fully helical structure with a kink stabilized by a 1-4 hydrogen bond between the Leu\(^12\) CO and Val\(^1\) NH. The helical structure is composed of \(3_{10}\)-helix in the N-terminal first turn and the C-terminal moiety following Leu\(^12\), and \(\alpha\)-helix in the middle region. In contrast, the Aib\(^14\) derivative predominantly has a straight \(\alpha\)-helical structure except for a \(3_{10}\)-helix region in the N-terminal first turn.

Key words Trichoderma polysporum; peptaibol; trichosporin; \(\alpha\)-aminoisobutyric acid; ion-channel

Trichosporin Bs (TS-Bs)\(^3-4\) are peptides isolated from the culture broth of the fungus Trichoderma polysporum. (LINK to PERSON) Rifai (strain TMI 60146). These peptides contain a high proportion of \(\alpha\)-aminoisobutyric acid (Aib) and their N- and C-terminal residues are protected by an acetyl group (Ac) and phenylalaninol (Pheol), respectively. On the basis of these characteristics, TS-Bs are members of the peptaibol family.\(^5\) They show biological activities such as uncoupling of oxidative phosphorylation in rat liver mitochondria\(^6\) and induction of catecholamine secretion from bovine adrenal medullary chromaffin cells.\(^7\) These bioactivities are related to an alteration of ion permeability through biomembranes, suggesting that TS-Bs form ion-channels in the membranes or modify the membranes. Recently, we found that TS-B-Vla, one of the major components of TS-Bs, forms ion-channels in planar lipid bilayers,\(^8\) as other peptaibols do. Many peptaibols, e.g. alamethicins,\(^9\) suzukacilins,\(^10\) paracelsins,\(^5\) trichorzianines,\(^11\) hypelcins,\(^12\) and trichocellins,\(^13\) have a Pro residue at the seventh position from the C-terminal. This Pro residue generates a kinked structure\(^14-16\) which is supposed to play an important role in voltage-gated ion-channel formation.\(^14\)

To examine the role of Pro\(^14\) in the structure and function of peptaibols, TS-B-Vla was derived to Aib\(^14\)-TS-B-Vla, in which Pro in TS-B-Vla is replaced by Aib.\(^17\) TS-B-Vla and Aib\(^14\)-TS-B-Vla have the following primary structures: TS-B-Vla (Aib\(^14\)-TS-B-Vla), Ac-Aib-Ala-Aib-Ala-Aib-Gln-Aib-Ile-Aib-Gly-Leu-Aib-Pro(Aib)-Val-Aib-Aib-Gln-Gln-Pheol. In this paper, we describe the solution structures of these peptides.

Materials and Methods

Synthetic TS-B-Vla and Aib\(^14\)-TS-B-Vla\(^17\) were used for the experiments. CD spectra were recorded at 23°C on a JASCO J-720 spectropolarimeter using a 1-mm path cell. For NMR measurements, samples were dissolved in CD\(\text{OH}\), containing TMS as an internal standard. All \(^1\)H-NMR spectra were recorded by a Bruker AM-600 (600 MHz) spectrometer. DQF-COSY spectra\(^18\) were measured in the phase-sensitive mode with selective saturation of the OH resonance of the solvent at all times. A total of 512 \(t_1\)-values were recorded, with 2048 data points and 32 scans for each \(t_1\). NOESY spectra\(^19\) were measured in the phase-sensitive mode with selective saturation of the OH resonance of the solvent at all times except during the detection periods. A total of 512 \(t_2\)-values were recorded, with 2048 data points and 64 scans for each \(t_1\). The data matrices of DQF-COSY and NOESY were filtered by shifted squared sine-bell multiplication in the \(t_1\) and \(t_2\) dimensions and zero-filled to 2048 data points in the \(t_1\) dimension; spectral width 6000 Hz; relaxation delay time 2.4 s. For NOESY, the mixing time was 300 ms with 10% random variation. The spectra were unsymmetrized. Under the NMR conditions, there was no evidence for peptide aggregation: no broadening of signals or significant variations of chemical shifts were detectable in the concentration range from 5 to 40 mM and in the temperature range from 268 to 307 K.

Results and Discussion

As shown in Fig. 1, the CD spectra of TS-B-Vla and Aib\(^14\)-TS-B-Vla in methanol were characteristic of right-handed helices. The ellipticities of Aib\(^14\)-TS-B-Vla at 208 and 222 nm were greater than those of TS-B-Vla, indicating that the replacement of Pro with Aib increased the helical content of the molecule. In our previous work, a similar increment of helicity was observed in des-Pro\(^14\)-TS-B-V,\(^16\) a synthetic 19-residue analogue of TS-B-V without the Pro\(^14\) residue. These increments of helicity are consistent with the fact that Aib promotes helical conformation and Pro often breaks it.\(^20,21\)

---

Fig. 1. CD Spectra of TS-B-Vla and Aib\(^14\)-TS-B-Vla in MeOH (25°C)

Molecular ellipticity, \(\theta\) (deg cm\(^2\) dmol\(^{-1}\)), at 208 and 222 nm: -3.415 \times 10^5 and -2.639 \times 10^5 (TS-B-Vla), -4.219 \times 10^5 and -3.706 \times 10^5 (Aib\(^14\)-TS-B-Vla).

© 1995 Pharmaceutical Society of Japan

* To whom correspondence should be addressed.
The secondary structure of a protein can be estimated from the "chemical shift index," the difference between the CaH chemical shift in a protein structure and that in a random coil. When the chemical shift indices are consecutively less than -0.1 ppm for more than four residues, the structure in that part is considered to be a helix, while more than three consecutive values greater than +0.1 ppm suggest a β-strand. Termination points of helices or β-strands are indicated by the appearance of chemical shift indices that are opposite in magnitude to those of the corresponding secondary structure or by the first appearance of two consecutive values within ±0.1 ppm. Figure 2 shows the chemical shift indices calculated for TS-B-V1a and Aib14-TS-B-V1a. Because of the lack of an α proton in Aib, the values for Aib residues are not marked. The results suggested that a helical structure in the N-terminal moiety of TS-B-V1a terminated at Gly11 and another helical structure was located in the C-terminal part from Val15. On the other hand, all the chemical shift index values of Aib14-TS-B-V1a were less than -0.1 ppm, suggesting that the Aib14-TS-B-V1a molecule has a regular helical structure all over the sequence. These structures for TS-B-V1a and Aib14-TS-B-V1a were in agreement with those obtained from J values reported previously.

In order to obtain detailed structural information, NOEY spectra of both peptides (40 nm) were recorded in CD3OH (Fig. 3). In the spectra for both peptides, successive NOEs [δNN (i, i+1)-type] and other diagnostic NOEs [δNN (i, i+1), δNN (i, i+3), δNN (i, i+3), δNN (i, i+1)-type] strongly suggested overall helical structure (Fig. 4). Characteristic NOEs [δNN (i, i+4), δNN (i, i+3), and δNN (i, i+2)-type] were also observed in both peptides. These NOEs are generally used to distinguish between α-helix and 310-helix: in an ideal α-helical peptide, δNN (i, i+4) and δNN (i, i+3)-type NOEs are stronger and δNN (i, i+2)-type NOEs are weaker or cannot be observed, while in an ideal 310-helical structure, stronger δNN (i, i+2)-type NOEs are observed. In the N-terminal region of TS-B-V1a, a strong δNN (i, i+2) (between the acetyl CH3 proton and the Ala2 NH proton) and a strong δNN (i, i+4) (between the acetyl CH3 proton and the Ala4 NH proton) were observed spontaneously (Figs. 3a, 4a). Therefore, the N-terminal first turn of TS-B-V1a was considered as a mixed conformation of 310- and α-helix. In the region from position 4 of TS-B-V1a, a stretch of α-helix was indicated by strong or medium [δNN (i, i+4), i=4, 9] and [δNN (i, i+3), i=4, 9], and terminated at position 11, because the δNN (i, i+4) between the Gly11 NH and Val15 NH, or between the Leu12 NH and Aib14 NH were absent (Figs. 3a, 4a). In the C-terminal region following Pro14 of TS-B-V1a, [δNN (i, i+2), i=14, 15] were much stronger than [δNN (i, i+4), i=14, 15] (Figs. 3a, 4a). Therefore, the C-terminal region (positions 14 to 20) of TS-B-V1a takes predominantly a 310-helical conformation. However, the weaker [δNN (i, i+4), i=14, 15] in this 310-helical region suggested that the 310-helix is not tightly bound and has a larger radius and a smaller pitch than those of the ideal 310-helix. It is interesting that in our previous work on TS-B-V, a member of TS-Bs, we found that the C-terminal moiety has α-helical conformation. The conformational difference between TS-B-V1a and TS-B-V could be due to the influence of position 3 (Aib in TS-B-V1a, Ala in TS-B-V).

In Aib14-TS-B-V1a, the N-terminal first turn has a mixed 310- and α-helical structure, as in TS-B-V1a (Figs. 3b, 4b). In the other part, strong or medium δNN (i, i+4) and δNN (i, i+3) cross peaks were observed, indicating that α-helical structure is predominant in this region. Thus, the conformation of Aib14-TS-B-V1a can be regarded as a straight cylinder.

The kinetics of proton-deuterium (H-D) exchange between backbone NH and deuterium ions in the solvent provides information on the flexibility of backbone hydrogen-bonds (H-bond). The H-D exchange rates of both peptides are shown in Fig. 5. The exchange rates of positions 1 to 12 are similar for both peptides, suggesting that the conformations of these peptides are almost identical in this region. The very high exchange rates for the N-terminal Aib1 NH and Ala2 NH (Fig. 5a) indicated that these protons are not involved in intramolecular H-bonding and are always exposed to the solvent. The low rates for the Aib3 NHs and Ala4 NHs of both peptides supported the presence of two types of H-bonds, as indicated by the NOE data: i) a 1→4 H-bond between the acetyl CO and the Aib3 NH (3,10-helical turn) and ii) a 1→5 H-bond between the acetyl CO and the Ala4 NH (α-helical turn). The rates for positions 5 to 20 of both peptides were low enough to imply that all the NHs in this region participate in inter-residue H-bonding. The Val15 NH and the Aib16 NH of TS-B-V1a should participate in H-bonding with the Leu12 CO and Aib13 CO, respectively, because the 1→5 H-bonding between the Gly11 CO and Val15 NH or between the Leu12 CO and Aib16 NH is ruled out by the NOE data. However, the H-D exchange rate for the Aib16 NH of TS-B-V1a was faster than those of other NHs except for the N-terminal NHs (Fig. 5), suggesting that the H-bonding of this NH is not so tight and this residue may be exposed to the solvent. Accordingly, the 1→4 H-bonding between the Leu12 CO and the Val15 NH would stabilize the Pro-
Fig. 3. Parts of the 600 MHz NOESY Spectra of 40 mM TS-B-VIa (a) and 40 mM Aib^{14}TS-B-VIa (b) in CD_{3}OD at 268 K, with a Mixing Time of 300 ms.

NOE cross-peaks [d_{ij}(i, j+1) or d_{ij}(i, j+4)] are labeled with the numbers of two residues involving acetyl CH_{3} (0). In order to enhance the NOEs, NOESY spectra were recorded at low temperature (268 K) and the signals have been assigned by means of the same procedures as described in the previous paper.\textsuperscript{1b}

(a) TS-B-VIa

| Residues | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| d_{01}(i, i+1) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+1) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+2) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+3) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+4) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+5) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

(b) Aib^{14}TS-B-VIa

| Residues | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
| d_{01}(i, i+1) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+1) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+2) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+3) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+4) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |
| d_{01}(i, i+5) |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |   |

Fig. 4. Inter-Residual NOEs Observed in CD_{3}OD at 268 K for TS-B-VIa (a) and Aib^{14}TS-B-VIa (b)

The observed NOEs are classified based on the cross-peak counter levels (represented by the thickness of the lines). Residue 0 represents the acetyl group.
Fig. 5. Hydrogen-Deuterium (H-D) Exchange Rates of the Amide Protons at 293 K

Exchanging rates (kex) are defined as the slope of semilogarithmic plots of the decay of amide signal intensity versus the time (h) after dissolving the peptides in CD3OD. Because of an overlap of the signals, the rate of Ala4NH for TS-B-Vla was not detected. Note that the scales of kex, for positions 1 to 3 (a) and for positions 4 to 20 (b) are different.

![Graph showing H-D exchange rates](image)

**Fig. 6. Temperature Coefficients of NH Chemical Shifts for TS-B-Vla and Aib14,TS-B-Vla Obtained over the Range of 286–307 K in CD3OH**

Positive $\Delta\delta/\Delta T$ (ppb/K) values represent upfield shift of the NH signals with increasing temperature.

![Graph showing temperature coefficients](image)

kinked structure in TS-B-Vla, minimizing repulsion between the imino ring of Pro14 and the Gly11 CO-14,16

The NH thermal coefficient values for the two peptides are shown in Fig. 6. Most values are small and negative (upfield shift with temperature increase). The values for both peptides at the N-terminal residues (Aib1 NH-Aib10 NH) were similar, supporting the strong similarity of conformations of the two peptides in this region. The larger values for the Aib1 NH and the Ala2 NH of both peptides indicate that these NHs are not involved in the intramolecular H-bonding, but are exposed to the solvent. The Aib3 NHs of both peptides and the Val15 NH and the Aib16 NH of TS-B-Vla show significantly small values. These NHs are involved in 1-4 H-bonding at the turning points from 310-helix to $\alpha$-helix or $\alpha$-helix to 310-helix. The small values would be due to the specific effects of these locations. The inter-residual H-bonding schemes proposed for TS-B-Vla and Aib14,TS-B-Vla are illustrated in Fig. 7.

The Pro14→Aib14 substitution of TS-B-Vla alters its C-terminal (Leu12 to Pheol29) 310-helical conformation to $\alpha$-helical conformation. Conversion of $\alpha$-helix-Pro310-helix structure to a long $\alpha$-helix was also observed in phage T4 lysozyme in which the Pro was replaced with any of seven other amino acids.20 Several conformational studies of Aib-rich peptides have revealed that these peptides with less than eight residues preferentially form 310-helix, while $\alpha$-helix was preferred by longer ones.21,29 These observations are in agreement with the result that the 310-helix appears in the small C-terminal moiety (seven residues) following the kink around Pro14 in TS-B-Vla, whereas, owing to the lack of the Pro-kinked structure, $\alpha$-helix is predominant in Aib14,TS-B-Vla.

Both alamethicin9 and TS-B-V16 form an $\alpha$-helix-kink (Pro14)-$\alpha$-helix structure. In this structure, the polar Gln side chains and COs (Aib10 and Gly11) not participating in inter-residual H-bonds align on one side of the helix. A single pore which is formed by a bundle of such helices seems to be the most probable form of voltage-gated ion-channel (Fig. 8a).14 On the other hand, TS-B-Vla forms an $\alpha$-helix-kink (Pro14)-310-helix structure. In this structure, the polar side chains of Gln18 and Gln19 should be located on the opposite side to the polar surface (CONHs of Gln7 and COs of Aib10 and Gly11) of the N-terminal helical domain. If TS-B-Vla forms ion-channels by the bundle model, the conformation of TS-B-Vla seems disadvantageous to the amphiphilic properties and to the stabilization of the bundle of helices. However, considering that the bundle is placed perpendicular to the lipid bilayers, a bundle of $\alpha$-helix-kink-310-helix could be stabilized by the interaction between C-terminal Gln side-chains and the polar region of lipid bilayers (Fig. 8b). Therefore, these polar side-chains could act as anchoring groups to the membrane surface.

Since Aib14,TS-B-Vla forms an amphiphilic helix without a kinked structure, a bundle of such helices would suffer from steric hindrance owing to the bulky C-terminal residues. Furthermore, the bundle of Aib14,TS-B-Vla lacks solvent-accessible carbonyls, such as Aib10 CO and Gly11 CO in TS-B-Vla, in the polar surface of the pore.
Fig. 7. Predominant Intramolecular Hydrogen Bonding Patterns for TS-B-Vla (a) and Aib14-TS-B-Vla (b).

The one-letter code of amino acid residues is used with U = Aib, F = Pheol.

Fig. 8. Ion Channel Models Proposed for TS-B-Vla and Aib14-TS-B-Vla.

Polar parts (NHs of Aib1 and Ala2; 4CONHs of Glu3, Gln5, and Gly6; OH of Pheol10; COs of Aib10 and Gly11) of the peptide monomers are shown by oblique lines. The 3-helix-kink-3-helix motif structure of alumethin and TS-B-Y (a) and the 3-helix-kink-3-helix structure of TS-B-Vla (b) each form a funnel-shaped channel from a bundle of monomers. The Aib14-TS-B-Vla channel is a bundle of straight cylindrical monomers (c).

Fig. 9. Effects of TS-B-Vla and Aib14-TS-B-Vla on Catecholamine Secretion from Cultured Bovine Adrenal Chromaffin Cells.

The secretion was measured as previously described. Adrenal chromaffin cells were isolated from bovine adrenal glands by collagenase digestion. The cells in modified Eagle’s minimum essential medium were maintained at 37°C in a CO2 incubator and were used for experiments after 4 days of cultivating. The cells were incubated for 10 min at 37°C with 5 μM TS-B-Vla or Aib14-TS-B-Vla in Ca2+-containing or Ca2+-free medium (plus EGTA). Catecholamine secretion is shown as a percentage of total cellular catecholamine content (19 ± 10 nmol). Data are means ± standard deviations from three experiments. The secretion was completely dependent on external Ca2+.

Consequently, the channel of Aib14-TS-B-Vla would be more unstable than that of TS-B-Vla (Fig. 8c). Indeed, the catecholamine secretion-inducing activity of Aib14-TS-B-Vla is less than that of TS-B-Vla, suggesting that the channel-forming ability of Aib14-TS-B-Vla is lower than that of TS-B-Vla in the biomembranes (Fig. 9). The ion-channel-forming properties of these peptides will be discussed elsewhere.

Acknowledgements This work was supported in part by Grants-in-Aid for Scientific Research (05453180, 06303014, 06680558, and 06780467) from the Ministry of Education, Science and Culture of Japan.

References and Notes
2) The following abbreviations are used: CD = circular dichroism; DQF-COSY = double quantum filtered correlation spectroscopy; EPGA = ethylene glycol bis[β-aminoethyl ether]-N,N,N′,N′-tetraacetic acid; NOE = nuclear Overhauser effect; NOEY = nuclear Overhauser enhancement spectroscopy.