Solution Structure of a Syndecan-4 Cytoplasmic Domain and Its Interaction with Phosphatidylinositol 4,5-Bisphosphate*

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Syndecan-4, a transmembrane heparan sulfate proteoglycan, is a coreceptor with integrins in cell adhesion. It has been suggested to form a ternary signaling complex with protein kinase C and phosphatidylinositol 4,5-bisphosphate (PIP2). Syndecans each have a unique, central, and variable (V) region in their cytoplasmic domains, and that of syndecan-4 is critical to its interaction with protein kinase C and PIP2. Two oligopeptides corresponding to the variable region (4V) and whole domain (4L) of syndecan-4 cytoplasmic domain were synthesized for nuclear magnetic resonance (NMR) studies. Data from NMR and circular dichroism indicate that the cytoplasmic domain undergoes a conformational transition and forms a symmetric dimer in the presence of phospholipid activator PIP2. The solution conformations of both free and PIP2-complexed 4V have been determined by two-dimensional NMR spectroscopy and dynamical simulated annealing calculations. The 4V peptide in the presence of PIP2 formed a compact dimer with two twisted strands packed parallel to each other and the exposed surface of the dimer consisted of highly charged and polar residues. The overall three-dimensional structure in solution exhibits a twisted clamp shape having a cavity in the center of dimeric interface. In addition, it has been observed that the syndecan-4V strongly interacts not only with fatty acyl groups but also the anionic head group of PIP2. These findings reveal that PIP2 promotes oligomerization of syndecan-4 cytoplasmic domain for transmembrane signaling and cell-matrix adhesion.

Cell adhesion mediated by cell surface receptors triggers signal transduction cascades. Integrins, which are well characterized transmembrane receptors, are known to be involved in several cell regulatory mechanism including cell proliferation, morphology, and motility. During cell-matrix interaction, integrin clustering activates tyrosine kinases (1–3). Adhesion of several cell types to extracellular matrix molecules such as fibronectin involves both integrin- and heparin-binding domains, where assays were performed in the absence of serum and protein synthesis (3–11).

The specific interactions of the cytoplasmic domain of each integrin member are critical to trigger the downstream signal cascades. Although the functions of these interactions are not clear, some kinases such as focal adhesion kinase and integrin-linked kinase, and structural proteins such as α-actinin, talin, and filamin, are known to interact with the cytoplasmic domain of β1 integrin subunit. The four mammalian syndecans are type I glycoproteins that form dimers or higher order multimers. Oligomerization apparently involves their highly homologous transmembrane domains as well as regions of their cytoplasmic domains (12–14). The central region of each syndecan cytoplasmic domain is unique, and has been termed the variable (V)1 region, flanked N- and C-terminally by constant (C1 and C2) regions (12, 13). Syndecan-4 is the only family member that is a widespread focal adhesion component, potentially functioning as a coreceptor in integrin-mediated adhesion. It readily forms oligomers that may be required for its function (12, 13).

Phosphatidylinositol 4,5-bisphosphate (PIP2) plays important roles in signal transduction, since PIP2 is hydrolyzed by phospholipase Cγ to generate two intracellular messengers: inositol 1,4,5-triphosphate, which mobilizes Ca2+, and diacylglycerol, which is a physiological activator of protein kinase C (PKC) (15–19). However, in addition to its function as precursor for other second messenger, PIP2 may also act as a second messenger by directly or indirectly regulating several proteins including PKC, phospholipase Cγ, phosphatidylinositol 3-kinase. PIP2 binds the cytoskeletal proteins gelsolin, profilin, and vinculin and regulates their interaction with actin (20–23). Recently, some β1 integrin cytoplasmic domains have been demonstrated to affect phosphatidylinositol 4,5-kinase, possibly regulating PIP3 synthesis in vivo (24–25). Indeed, it is known that PIP2 is accumulated in the cytoplasmic face of plasma membrane during cell adhesion and it may be regulated by G-proteins such as Rac and Rho, which may activate phosphatidylinositol 4,5-kinase (26, 27). PIP2 is also known to be involved in focal adhesion and stress fiber formation (3), probably through its interactions with focal adhesion components such as vinculin, α-actinin, and syndecan-4.

Focal adhesion formation is dependent not only on integrins, but syndecans (most likely syndecan-4), which may act as coreceptors (2, 8, 9). It has been previously shown that syndecan and PKC are involved in the focal adhesion formation, and syndecan-4 regulates the distribution and activity of classical PKC (cPKC). In addition, it has been suggested the interaction of syndecan-4 cytoplasmic domain and cPKC depends on cPKC

1 The abbreviations used are: V, variable; C, constant; PIP2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, total correlation spectroscopy; DQF, double quantum filtered; COSY, correlated spectroscopy; SA, simulated annealing; cPKC, classical PKC.
activity. Recently, we have reported that PIP2 interacts specifically with the cytoplasmic domain of syndecan-4, and together with PIP2, syndecan-4 cytoplasmic domain potentiated PKC activity (28). It was also shown that PIP2 regulates the interaction of the cytoplasmic domain of syndecan-4 with PKCa and potentiation activity of syndecan-4 cytoplasmic domain on PKCa (28). As we suggested previously, syndecan-4 and PIP2 together with PKC may form a ternary complex. PKC is activated through this complex, which may then phosphorylate cytoplasmic proteins involved in focal adhesion formation. Here, we present the solution structures of syndecan-4 cytoplasmic core domain both with and without activator PIP2 by two-dimensional nuclear magnetic resonance spectroscopy and dynamical simulated annealing calculations.

**EXPERIMENTAL PROCEDURES**

**Sample Preparations**—Peptides of both the whole domain with RMKKDEGSYDLGKKPIYKAPTNEFYA sequence corresponding to residues 170–197 (4L) and the variable region with LGKPIYKKKAPTNEFYA corresponding to residues 181–190 (4V) of the chicken syndecan-4 cytoplasmic domain were synthesized using an improved version of the solid phase method on a model 431A peptide synthesizer (Applied Biosystems Inc.). The peptide sequences are identical to those of human and rat syndecan-4 cytoplasmic domain. Peptides were purified by reversed-phase liquid chromatography using a Vydac 218TP152050 C18 column on a Waters Delta Prep 4000 system. Purification was achieved by equilibrating the column with 0.1% trifluoroacetic acid in water and developing with a linear gradient of acetonitrile.

The free peptide samples for NMR measurements were prepared by dissolving in 90% H2O, 10% D2O or 99.9% D2O solution at pH value of 7.4 with 50 mM sodium phosphate buffer. The final peptide concentration was 2–4 mM in 0.5 mM of buffer solution. PIP2 was purchased from Sigma. For NMR measurements of PIP2-peptide complex, 10–50 μL of concentrated PIP2 solution was titrated to free peptide up to a maximum ratio of 1:1 (PIP2:peptide). The final pH value of 7.0 was adjusted by adding 0.1 M HCI solution before NMR experiments.

**Circular Dichroism**—CD data were collected on a Jasco J-715 spectropolarimeter using 1.0-mm path length cells with scan speed of 500 min/1 nm, 1-nm bandwidth, and 32 accumulations. Both free and PIP2-complexed samples of 4L and 4V peptides were prepared as volumes of 8 μL in H2O solution, and CD data were collected for temperatures ranges of 20–70 °C. A standard noise reduction procedure was used to prepare final spectrum.

**NMR Spectroscopy**—All NMR experiments were performed on a Bruker DMX600 spectrometer in quadrature detection mode equipped with an SGI INDY computer. All data were collected at either 10 °C or 20 °C, and the strong solvent resonance was suppressed by water-gated pulse sequence combined with pulsed-field gradient pulses. The temperature was calibrated using a methanol standard (29). A series of one-dimensional NMR spectra were recorded for both 4V and 4L peptide with concentration ranges of 0–2 mM PIP2. All of the two-dimensional NMR measurements were performed on both free and peptide-PIP2 (2:1) samples based on results from PIP2 titration analysis. Mixing times of 100, 200, 300, 400, 500, and 800 ms were used in collecting NOE spectra for both free and PIP2 complex samples. Total correlation spectroscopy (TOCSY) data were also recorded in both H2O and D2O solutions with a mixing time of 78 ms using MLEV17 spin lock pulses (30). Double quantum-filtered (DQF) COSY spectra (31) were collected in H2O solution. All data were recorded in the phase-sensitive mode using the time proportional phase incrementation method (32) with 2048 data points in the acquisition domain and 512 or 256 in the time domain. Two-dimensional NOESY (33) experiments were also performed to identify slowly exchanging amide hydrogens on a freshly prepared D2O solution after lyophilization of an H2O sample. Small flexible linear peptides such as 4V have relatively short correlation times compared with globular proteins. As a result, longer mixing times or rotating frame Overhauser effect spin lock times are typically needed before NOE or rotating frame Overhauser effect cross-peaks begin to build up in the spectrum. From the NOESY growth curve, we have ascertained that spin-diffusion effects are not significant even for 400–800 ms mixing times. The interesting cross-peaks observed in the NOESY spectra are due to direct effects since no other cross-peaks from protons that could mediate the intensity through spin diffusion were observed in the spectra. Long mixing times in the range of 400–800 ms have typically been used for small peptides.

All NMR data were processed using nmrPipe (Biosym/Molecular Simulations Inc.) or Bruker XWIN-NMR (Bruker Instruments) software on SGI Indigo2 workstation. Prior to Fourier transformation in the t1 dimension (34), the first row was half-weighted to suppress t1 ridges. The DQF-COSY data were processed to 8192 × 1024 data matrices to obtain a maximum digital resolution for coupling constant measurements. The proton chemical shifts were referenced with internal sodium 4,4-dimethyl-4-silapentane 1-sulfonate.

**Molecular Modeling Calculations**—All calculations were performed for syndecan-4V using XPLOR 3.1 (Biosym/Molecular Simulations, Inc) on an SGI Indigo2 workstation using the topology and parameter files of topallhdg.pro and parallhdg.pro. Monomer 4V structures were generated with random backbone dihedral angles and used as starting structures for simulated annealing (SA) calculations (35–38). The procedure by Nilges (35) was also used for symmetric dimer generations with minor modifications described by Lee et al. (39) as follows. Symmetric dimers were generated by duplications of the random coordinates. The combined use of XPLOR noncrystallographic symmetry and symmetry pseudo-NOE term were served to satisfy monomer symmetry as described by Nilges (35). The modeling protocol used by us consists of two separate stages: (i) initial and extensive regulation and (ii) simulated annealing and refinement. Regulation procedures were cycled twice to satisfy both experimental constraints and noncrystallographic symmetry parameters, followed by simulated annealing and refinement. The potential energy function consisted of covalent, repulsion, NOE, and...
torsional angle terms. The target function forms of NOE and torsional angles are the same as used by Driscoll et al. (40). A total of 116 distance restraints and 16 torsional angle constraints were used for structural calculations. All NOEs were classified and converted to distance constraints as strong (1.8–2.7 Å), medium (1.8–3.3 Å), and weak (1.8–5.0 Å) based on their intensities of NOESY spectrum. Corrections for pseudoatom representations were used for non-stereo specifically assigned methylene, methyl group, and tyrosine ring protons (32). Dihedral angle restraints were derived from measured $^3J_{HN-HN}$ coupling constants in DQF-COSY spectra in H$_2$O solution (41, 42). The structure

FIG. 2. A, proton one-dimensional NMR spectra of 4V peptide with addition of 0 mg (a), 0.5 mg (b), and 1.0 mg (c) of PIP$_2$, respectively. The molar ratios of peptide to PIP$_2$ are indicated on the left side of the spectra. B, proton one-dimensional NMR spectra of 4L with PIP$_2$ of 0 mg (a), 0.25 mg (b), 0.33 mg (c), and 0.5 mg (d), respectively.
The CD spectra of the 4V peptide (sequence LGKKPIYKKA) at pH 7.4 and 25 °C were consistent with a random coil characteristics (43). However, the CD spectra obtained from 4V peptide complexed with PIP2 were different from that of peptide alone, having a small positive mean residue ellipticity at 222 nm. The same spectral changes of 4L peptide were observed in the presence of PIP2. The CD data suggested that the structural transitions of both peptides were occurred in the presence of PIP2. Fig. 1 shows that the positive ellipticity intensity of 4V-PIP2 complex at 222 nm decreased gradually at temperature ranges of 20–60 °C, indicating a slow process of dissociation.

NMR Resonance Assignments and Secondary Structures—The one-dimensional proton NMR spectra of both 4V and 4L peptides are shown in Fig. 2. The spectra have demonstrated that both peptides produced considerable changes in the whole peptide resonance as well as in linewidths of resonance throughout PIP2 titration. Glycine was easily identified by the distinctive fingerprint pattern in the H2O COSY spectra, and the lone alanine residue was identified from connectivities in TOCSY spectra by its characteristic methyl resonance. One AMX spin system from the tyrosine residue was easily identified in TOCSY spectra. These preliminary resonance assignments served as starting points for the sequence-specific assignment procedure (44).

Table I.

| Statistics                  | (SÅ) | (SÅ) | (SÅ) |
|-----------------------------|------|------|------|
| A. Root-mean-square deviations from experimental distance restraints (Å) |      |      |      |
| All (116)                   | 0.0349 | 0.0399 |      |
| Sequential ( | | | |
| i–j | = 1) (32) | 0.0391 | 0.0227 |      |
| Intraresidue (80)           | 0.0341 | 0.0355 |      |
| Interresidue (36)           | 0.0353 | 0.0483 |      |
| B. Energies                 |      |      |      |
| E_total (kcal mol⁻¹)        | 77.20 | 55.61 |      |
| E_NOE(all) (kcal mol⁻¹)      | 7.19  | 8.65  |      |
| E_repel (kcal mol⁻¹)        | 48.12 | 29.16 |      |
| E_nuc (kcal mol⁻¹)          | 11.57 | 8.35  |      |
| E_improper (kcal mol⁻¹)     | 0.19  | 0.004 |      |
| C. Deviations from idealized covalent geometry |      |      |      |
| Bonds (Å)                   | 0.003283 | 0.0029 |      |
| Angles (degree)             | 0.673114 | 0.5002 |      |
| Improper (degree)           | 0.474157 | 0.3530 |      |

CD of Free and PIP2-complexed Peptide—The CD spectra of the 4V peptide (sequence LGKKPIYKKA) at pH 7.4 and 25 °C were consistent with a random coil characteristics (43). However, the CD spectra obtained from 4V peptide complexed with PIP2 were different from that of peptide alone, having a small positive mean residue ellipticity at 222 nm. The same spectral changes of 4L peptide were observed in the presence of PIP2. The CD data suggested that the structural transitions of both peptides were occurred in the presence of PIP2. The CD data suggested that the structural transitions of both peptides were occurred in the presence of PIP2. Fig. 1 shows that the positive ellipticity intensity of 4V-PIP2 complex at 222 nm decreased gradually at temperature ranges of 20–60 °C, indicating a slow process of dissociation.

NMR Resonance Assignments and Secondary Structures—The one-dimensional proton NMR spectra of both 4V and 4L peptides are shown in Fig. 2. The spectra have demonstrated that both peptides produced considerable changes in the whole peptide resonance as well as in linewidths of resonance throughout PIP2 titration. In particular, 4L peptide experiences a severe aggregation at ratio of 8:6 (4V:PIP2) (Fig. 2, B, d). There are only single glycine, tyrosine, and alanine residues in the 4V peptide sequence. Glycine was easily identified by the distinctive fingerprint pattern in the H2O COSY spectra, and the lone alanine residue was identified from connectivities in TOCSY spectra by its characteristic methyl resonance. One AMX spin system from the tyrosine residue was easily identified in TOCSY spectra. These preliminary resonance assignments served as starting points for the sequence-specific assignment procedure (44). Sequential resonance assignments were made based on two-dimensional TOCSY and NOESY spectra in 90% H2O, 10% D2O solution. Sequential assignments for the backbone protons were completed by following d_HN connectivities from NH-CαH COSY cross-peaks of previ-
uously identified amino acids. The side-chain proton chemical shifts were completed by TOCSY connectivities. The resonances of PIP₂ were identified from the previously published chemical shifts (45) and PIP₂ titration procedure (Fig. 2). Some of the PIP₂ resonance signals in 4V-PIP₂ complex were assigned from proton one-dimensional spectrum, followed by PIP₂ titration to 4V. Since most of PIP₂ resonances were severely overlapped with peptide signals, PIP₂ chemical shifts were identified from all assigned peptide resonances in two-dimensional TOCSY and NOESY spectra. All these sequential and medium NOE connectivities for both free and complex are summarized in Fig. 3. Most of the NOEs shown in Fig. 3 were measured at mixing times of 100–400 ms. For 4L peptide, since the backbone NMR resonances were severely overlapped due to 7 lysine residues in the sequence, not all NOEs from residues involved in the variable domain could be assigned completely.

The proton chemical shifts for the individual residues in free 4V peptide are in excellent agreement with the corresponding random coil values (46). As expected for a highly flexible peptide, NH–CαH coupling constants for all the residues are of the order of 6.5–8.0 Hz, suggesting retention of significant conformational flexibility. In addition, the sequential NOE intensities displayed a standard pattern of the preferred random coil conformation of the peptide. However, when peptide was titrated with PIP₂, both the chemical shifts and NOE patterns exhibited a significant change in the entire peptide sequence indicating a structural transition (Figs. 3 and 4). In addition, a number of intermolecular NOEs, which are NOEs between peptide and PIP₂ and intersubunit NOEs on the 4V dimer, were observed. The aliphatic proton resonance region of the two-dimensional NOESY spectrum of 4V-PIP₂ is shown in Fig. 5, displaying both intersubunit and intermolecular NOEs in the complex.

**Three-dimensional Structures**—It is of interest to compare the proton chemical shift differences between free and PIP₂ complex 4V. Fig. 4 shows that, whereas proton chemical shifts of the side chains showed not much changes, those of the backbone NH and CαH changed dramatically in N-terminal as well as C-terminal regions. The side-chain chemical shifts, especially Pro¹⁸⁵ and Ile¹⁸⁶ residues in the center of peptide, exhibited significant changes. These interesting results indicate that the 4V dimer has extensive intermolecular contacts not only at both peptide terminus but also in the middle of the peptide (Fig. 4). In addition, these results strongly suggest that, while the side-chain atoms would have close intersubunit contacts with each other in the center of peptide, the backbone atoms do so at peptide termini.

The solution structures for the 4V dimer were generated using the experimental constraints described above. A total of 90 starting substructures were used in the initial stage. After two cycles of regulation protocol, 60 structures that show no constraint violations greater than 0.5 Å for distances and 5° for torsional angles were identified. Among 60 structures, the 14 lowest energy structures (SAkr) were selected for detailed analysis (Table I). The structures are well defined with a root-mean-square deviation between backbone atom coordinates of 1.05 Å for all residues. The average structure was generated from the geometrical average from 14 structure coordinates and was subjected to restrained energy minimization to correct bond length and angle distortions. This average structure exhibited 0.59-Å root-mean-square deviation for backbone atoms with respect to 14 (SA)kr structures. A best fit superposition of
all final structures and the backbone conformation for average restrained energy minimized structure (\(\langle S A \rangle_{\alpha} \)) are shown in Fig. 6. The atomic average root-mean-square deviations of the final structures with respect to average restrained energy minimized structure are shown in Fig. 7. A Ramachandran plot (Fig. 8) indicates that \(\phi, \psi \) values of all 14 final NMR structures are distributed properly in energetically acceptable regions.

An inspection of the average energy-minimized structure in Fig. 6B clearly shows that major driving forces for dimer formation could be originated from hydrophobic interactions among side-chain atoms. 4V peptide in 4V-PIP\(_2\) complex forms a compact symmetric dimer with two extended strands twisted parallel each other. This compactness explains why 4V dimer dissociates slowly during the subunit exchange experiment as shown in Fig. 1. The exposed surface of the 4V consisted of very polar residues with basic side chains. Interestingly, the Pro\(^{185}\) residue that resides in the center of the cytoplasmic core has been shown to play a key role for twisted dimeric formation. No standard regular secondary structural elements were observed, as shown in Fig. 1. However, each monomer conformation exhibits similar to that of extended form with both N and C termini with kink. Strikingly, the overall three-dimensional structure of the 4V in solution demonstrates a twisted clamp shape having a cavity in the center of dimeric twist (Fig. 6C).

**Molecular Interaction with PIP\(_2\)**—A number of NOE contacts between 4V and PIP\(_2\) have been observed, which are NOEs between Pro\(^{185}\) ring protons of 4V and head group protons of PIP\(_2\), Ile\(^ {186}\) C\(^6\)H\(_3\) methyl group of 4V and fatty acyl group of PIP\(_2\), and C\(^2\)H\(_2\) of Lys\(^ {188}\) and olefinic protons of PIP\(_2\). These NOEs strongly suggest that both anionic head group and fatty acyl side chain of PIP\(_2\) have close contacts with center of the peptide. Table II summarizes NOEs between 4V and PIP\(_2\). Especially, a possible hydrogen bond between phosphatidylinositol 4-phosphate oxygen and Lys\(^ {188}\) eNH\(^ +\) of 4V was observed from modeling structure based on NMR data. Fig. 9 shows the averaged energy-minimized solution structure of 4V complexed with PIP\(_2\). The 4V dimer grips PIP\(_2\) having interactions with both head group and fatty acyl chains of PIP\(_2\). The solution structure clearly reveals that side chains of cytoplasmic core residues play an important role for PIP\(_2\) binding.

It has been suggested that the syndecan core proteins, which are highly conserved among the syndecan family, exhibit a propensity to form non-covalently linked dimers and higher order oligomers (12–14). It is also well known that oligomer formation is essential in protein kinase C interaction and activation (12). Recently, we have reported that SDS-resistant multimerization of syndecan-4 core protein correlates with PKC regulatory activity and variable region unique to syndecan-4 (4V) could potentiate phospholipid-induced activation of PKCa\(\beta\)\(\gamma\) (12, 13). In addition, it has been shown that a central penta-peptide KPIYK sequence is necessary for multimerization of syndecan-4 core protein, resulting in both direct activation of PKCa\(\beta\)\(\gamma\) and phospholipid-induced activation (12, 13).
Further, 4V or 4L peptides in the presence of PIP2 strongly activate PKCα (28). The data from NMR and gel filtration clearly support the hypothesis that PIP2 induces dimerization of both 4V and 4L peptides, and the induced dimer of 4V forms a compact structure bound to PIP2 (Fig. 2). We have recently reported that the peptide of whole cytoplasmic domain (4L) displayed less tendency to oligomerize than 4V in the absence of PIP2, resulting in reduced activity of 4L. This could be due to highly basic residues of the conserved (C1) region, which lies between the V region of the cytoplasmic domain and the membrane. These residues may decrease the self-association of the 4L peptide. However, very recently, we have shown that both 4V and 4L peptides exhibited essentially the same activity with respect to PIP2-mediated activation of PKC (28). Therefore, we concluded that the solution structure of 4L in the presence of PIP2 should resemble that of PIP2-4V complex; in other words, the molecular topology of 4L and 4V in the presence of PIP2 must be similar. Major interaction of 4V peptide with PIP2 occurs between aliphatic side chains of peptide and whole area of PIP2. No close contacts of peptide backbone to PIP2 has been observed. The other finding is that, even though PIP2 promotes association of 4V initially, further driving or stabilizing forces could be peptide-peptide interactions. The NMR structure clearly shows that peptide backbones of each monomer consists

![Image](image1.png)

**Fig. 7.** Distribution of the average atomic root-mean-square deviations with respect to average structure of the backbone (A) and side-chain (B) atoms in the 14 final simulated annealing structures for the 4V dimer.

![Image](image2.png)

**Fig. 8.** Distribution of all (φ,ψ) values for the final 14 simulated annealing structures of the 4V in 4V-PIP2 complex. The glycine residues are represented by open circles.

![Image](image3.png)

**Fig. 9.** A, model of 4V-PIP2 complex generated with Insight II (Biosym/Molecular Simulation Inc.). PIP2 (space-filling model) and 4V (van der Waals and ribbon representation) are shown in violet and red, respectively. B, electrostatic potential surface of 4V and space-filling model of PIP2 (yellow) in 4V-PIP2 complex. The negative electrostatic potential is represented in red, the positive in blue, and the neutral in white. The potential surface was calculated using the Delphi program (Biosym/Molecular Simulation Inc.).

| TABLE II | Intermolecular NOEs between 4V peptide and PIP2 |
|----------|---------------------------------------------|
| PIP2     | 4V  |
| R1-C5,6,8,9H | Pro185 C91 (m) | Ile186 C5H1 (m) | Lys188 C95H1 (s) |
| R1-C7H   | Pro185 C5H2 (m) | Pro185 C5H2 (w) | Ile186 C5H2 (w) |
| Ring-1’5/6’ | Ile186 C5H2 (m) |

*NOEs are classified as s (strong), m (medium), and w (weak) at mixing times of 100–400 ms at 25 °C. The numbering of PIP2 is based on IUPAC nomenclature.*
of intertwined circular shape and side chains provide additional stabilizing forces by hydrophobic interactions from an outward position. Surprisingly, two extended monomers form a quite stable twisted dimeric conformation, even if there is no hydrogen bonds between monomers. Since the calculated 14 structures (3A) based on NMR constraints exhibited a good convergence, we conclude that the 4V dimer has a stable and unique conformation in solution state in the presence of PIP2. This was also confirmed by melting experimental data based on circular dichroism (Fig. 1) and NMR linewidth analysis at temperature ranges of 20–70 °C (data not shown). We have recently demonstrated that 4V peptides of altered sequence have much reduced ability to regulate PKC activity (12, 13, 28). It was suggested that substitution of Lys184, Lys188, Pro185, Tyr187 with phenylalanine had a profound effect on PKC activation, it had the least effect on dimer formation. This is in very good agreement with solution structure analysis. Tyr187 appears not to be involved in oligomerization of 4V, and we hypothesize that since its side chain is mostly solvent-exposed, the hydroxyl group may be critical for contact with, and activation of, PKC. Structural studies of the 4V-PIP2-PKC ternary complex will provide this important information.

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