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Solution Structure of a GSK 3β Binding Motif, AXIN^{pep}

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Abstract : Axin is a scaffold protein of the APC/axin/GSK complex, binding to all of the other signalling components. Axin interacts with Glycogen synthase kinase 3β (GSK3β) and functions as a negative regulator of Wnt signalling pathways. To determine the solution structure of the GSK3β binding regions of the axin, we initiated NMR study of axin fragment comprising residues Val^{388} - Arg^{401} using circular dichroism (CD) and two-dimensional NMR spectroscopy. The CD spectra of axin^{pep} in the presence of 30% TFE displayed a standard α-helical conformation, exhibiting the bound structure of axin^{pep} to GSK3β. On the basis of experimental restraints including NOEs, and $^{3}J_{HN\alpha}$ coupling constants, the solution conformation of axin^{pep} was determined with program CNS. The 20 lowest energy structures were selected out of 50 final simulated-annealing structures in both water and TFE environment, respectively. The RMSDs for the 20 structures in TFE solution were 0.086 nm for backbone atoms and 0.195 nm for all heavy atoms, respectively. The Ramachandran plot indicates that the φ, ψ angles of the 20 final structures is properly distributed in energetically acceptable regions. Axin^{pep} in aqueous solution consists of a stable α-helix spanning residues from Glu^{391} to Val^{399}, which is an interacting motif with GSK3β.

Keywords : Axin, GSK3β, Circular Dichroism, NMR

INTRODUCTION

Axin consists of an N-terminal 'regulator of G protein signaling' domain and a C-termianl DIX domain. The N-terminal domain (RGS) that binds with adenomatous polyposis coli (APC), a separate binding domain for GSK3β and β-catenin in the center. A C-terminal DIX (found in Dvl and Axin) domain was related to Dvl binding and oligomerisation of axin.[1] Oligomerisation of axin plays an important role as a negative

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regulator of Wnt signalling.[2] Axin was originally identified from the characterization of the Fused locus, the disruption of which leads to duplication of axis and embryonic lethality. Indeed, when axin is injected into Xenopus embryos, most embryos develop with strong axial defects.[3-5]

Axin is a scaffold protein of the APC/axin/GSK complex, by binding to all of the other signalling components[6], and it regulates multiple signaling pathways. Especially, Axin interacts with Glycogen synthase kinase 3β (GSK3β) and functions as a negative regulator of Wnt signalling pathways.[7]

The axin^{pep} consisting of has been reported to bind as a single amphipathic α-helix, into a hydrophobic surface channel at GSK3β C-terminal helical domain.[8] To determine the solution conformation of the axin^{pep} fragment, we initiated NMR studies for a peptide comprising residues of Val^{388} to Arg^{401}.

MATERIALS AND METHODS

Peptide Synthesis and Purification

Axin^{pep} (^{388}VEPQKFAEELIHRLEAVQR^{401}) was synthesized commercially by Anygen Inc. (Gwangju, Korea). HPLC purification was performed with a Delta pak C18 HPLC column. The peptide was purified by using a reverse phase C18 column with 0.1% trifluoroacetic acid (TFA) in water, and developed with a linear gradient of acetonitrile. The purified peptide was characterized by high pressure liquid chromatography and mass spectrometry. The purity of the peptide was greater than 95% as determined by these methods.

NMR Sample Preparations

Samples for NMR experiments were 3mM concentration in 50mM sodium phosphate buffer pH values of 7.0 in 90% H_2O/10% ^2H_2O solution. NMR sample in 70% H_2O /30% 2,2,2-trifluoro-(d3)-ethanol(TFE) mixture (v/v) at pH 7.0 was prepared after lyophilization

of a sample in the aqueous solution.

Circular Dichroism Sprctroscopy

CD spectra of 40 μM axin^{pep} were measured in 50 mM sodium phosphate and various concentrations of TFE samples in pH 7.0 at 298 K on a Jasco 810 spectropolarimeter. Far-UV CD spectra were monitored from 190 to 250 nm by using quartz cell having path-length of 0.1 mm. Data were collected at 0.5 nm-interval and 5 scans were averaged with scan speed rate of 50 nm/min.

NMR Spectroscopy

NMR experiments were recorded at both 5 and 25℃ on a Bruker DRX-500 equipped with a triple-resonance probe with x, y, z- gradients. Two-dimensional (2D) NMR spectra were recorded in phase-sensitive mode by using time-proportional phase incrementation for quadrature detection in the t_1 domain. The 2D experiments such as double-quantum filtered COSY (DQF-COSY) [9], TOCSY[10] by using a MLEV-17 spin lock pulse sequence with a mixing time of 78ms and NOESY[11] with mixing times of 300 ~ 600 ms were performed. For DQF-COSY experiments, solvent suppression was achieved by using selective low-power irradiation of the water resonance during 2.0 s of relaxation delay. Solvent suppression for TOCSY and NOESY experiments was achieved by using a WATERGATE pulses.[12] All NMR spectra were acquired 2048 complex data points in t_2 and 256 increments in the t_1 dimension, with 32 scans per increment. The $^3J_{HN\alpha}$ coupling constants were measured from DQF-COSY spectra, strip-transformed to 8K × 1K.

NMR Data Processing

NMR data were processed on a Silicon Graphics Indigo^2 workstation by using NMRPIPE/NMRDRAW software (Biosym/Molecular Simulatons, Inc.) and XWINNMR (Bruker Instruments) software and analyzed by using the Sparky 3.95 program. The proton chemical shifts were expressed relative to the methyl resonance of the internal sodium 4,4-

dimethyl-4-silapentane-1-sulfonate at 0 ppm.

Structural Restraints and Structure Calculations

Structure calculations were performed by using a hybrid distance geometry and dynamical simulated-annealing protocol with CNS 1.0 program on a SGI Indigo^2 workstation. The methodology employed by us was similar to the original protocol of Clore and Gronenborn and their coworkers.[13-15] Distance geometry (DG) substructures were generated by using a subset of atoms in the peptide and followed a refinement protocol described in Lee et al.[16] The target function for molecular dynamics and energy minimization consisted of covalent structure, Van der Waals repulsion, NOEs and torsion angle constraints. The torsion angle and NOE constraints were represented by squar-well potentials. Distancs restraints were derived from the NOESY spectra recorded both in 90% H_2O/10% ^2H_2O and 70% H_2O/30% TFE solutions. A total of 191 NOEs derived from water sample was used for structure calculations. For TFE/water condition, a total of 276 NOEs, 3 backbone dihedral angles were used. Based on cross-peak intensities in the NOESY spectra with mixing times of 300, 600ms, the distance constrints were classified into three distance ranges, strong (1.8~2.7 Å), medium (1.8~3.3 Å) and weak (1.8~5.0 Å). Pseudoatom corrections were used for non-stereo-specifically assigned methylene protons, methyl groups and the ring protons of phenylalaine residue.[17] In addition, $^3J_{HN\alpha}$ coupling constant were used as -55±45° for a $^3J_{HN\alpha}$ less than 6Hz and -120±50° for a $^3J_{HN\alpha}$ greater than 8Hz. All modeling calculations were performed within CNS 1.0 program on a SGI Indigo^2 workstation. The visual analysis of resulting structures was carried out with MOLMOL[18] software running of Silicon Graphics workstations and RMSD values were also obtained from CNS 1.0 software.

RESULTS AND DISCUSSION

CD Spectroscopy

In order to investigate the conformational change induced by hydrophobic environment

such as TFE, the CD spectra were acquired at various concentrations of TFE. The CD spectra of 40 μM axin^{pep} were measured in 50 mM sodium phosphate and various concentrations of TFE samples in pH 7.0 at 298 K. Fig. 1 shows that the peptide contains a small population of helical conformation in TFE free and 10% (v/v) TFE/H_2O environment by the ellipticity at 222nm. However, the characteristic α-helical transition was clearly observed in 30% (v/v) TFE/H_2O solution. The spectrum showed clear double minima at 208 and 222 nm, indicating the existence of α-helix.

Resonance Assignments

The proton resonances were identified by using methods proposed by Wuthrich.[15] The first step was involved in the analysis of the TOCSY and NOESY experiments to identify the spin systems of particular amino acids. It was straightforward to assign two valines and

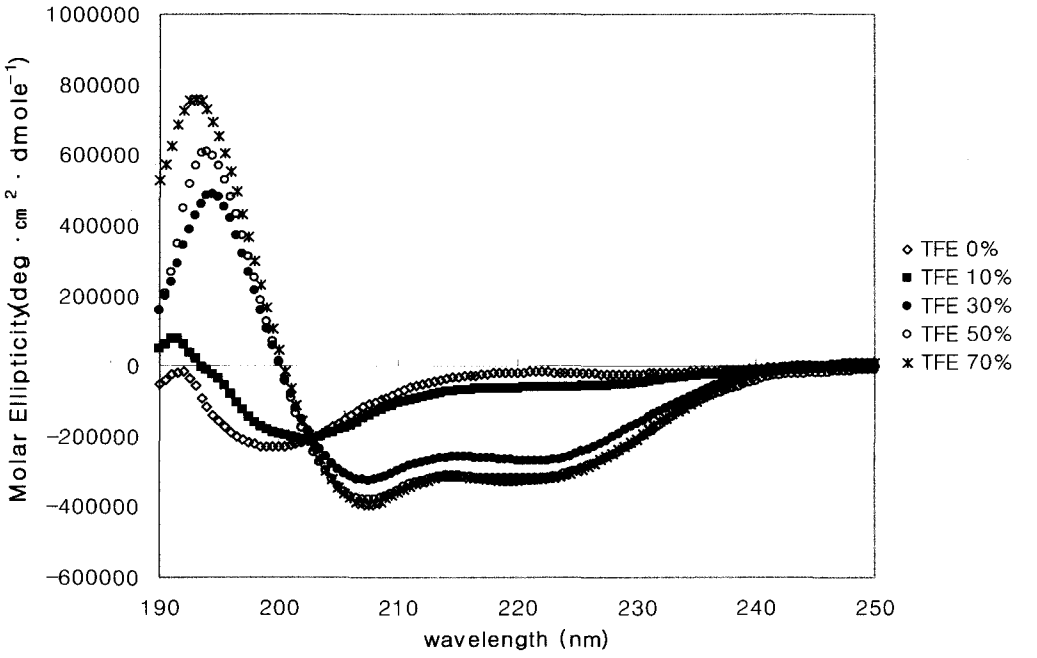

Fig. 1. CD spectra of axin^{pep} under various conditions: Axin^{pep} with different concentrations of trifluoroethanol (TFE) at pH 7.0, 25℃.

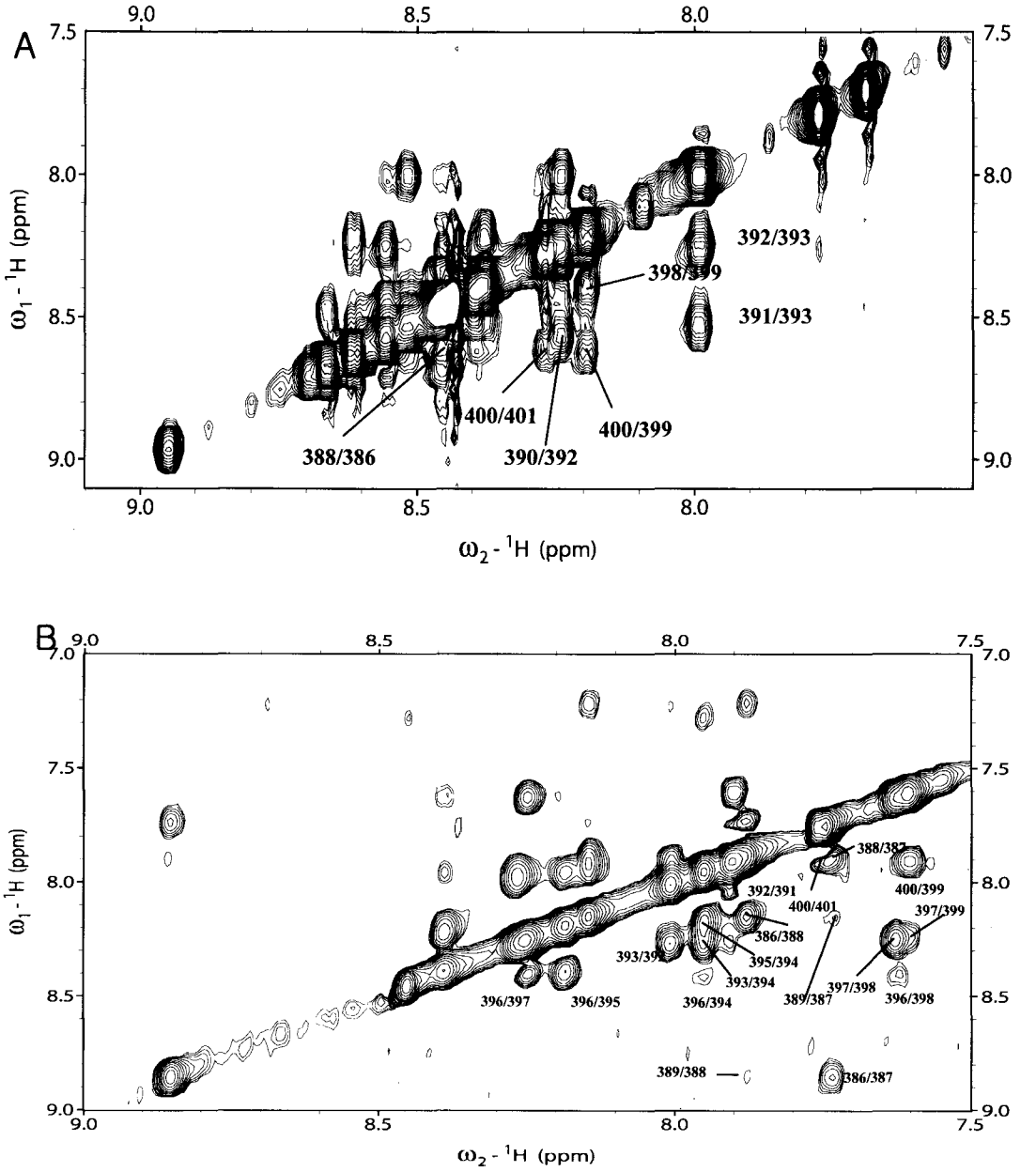

Fig. 2. 2D-NOESY spectra of axin^{pep} showing NH regions with mixing time of 300ms dissolved in (A) 90% H_2O/10% ^2H_2O solution at pH 7.0, 5℃ and (B) 70% H_2O/30% TFE solution at pH 7.0, 25℃.

two leucines because these residues showed characteristic resonances in the TOCSY spectra, and the NOESY spectra were served to identify all sequential connectivities for adjacent residues. The amide-amide regions of the NOESY spectrum in 90% H_2O/10% ^2H_2O solution and 70% H_2O/30% TFE solution are shown in Fig. 2. $Val^{388}C^{\alpha}H/Glu^{389}NH$ peak was not found in the NOESY spectrum, showing that N-terminal of $axin^{pep}$ could be flexible. However, a number of $d_{NN}(i, i+1)$ NOEs are clearly found at central residues of axin (Fig. 2B), supporting that the central region of $axin^{pep}$ forms a rigid structure. Fig. 3 presents a summary of NMR data containing sequential and short-range NOE contacts.

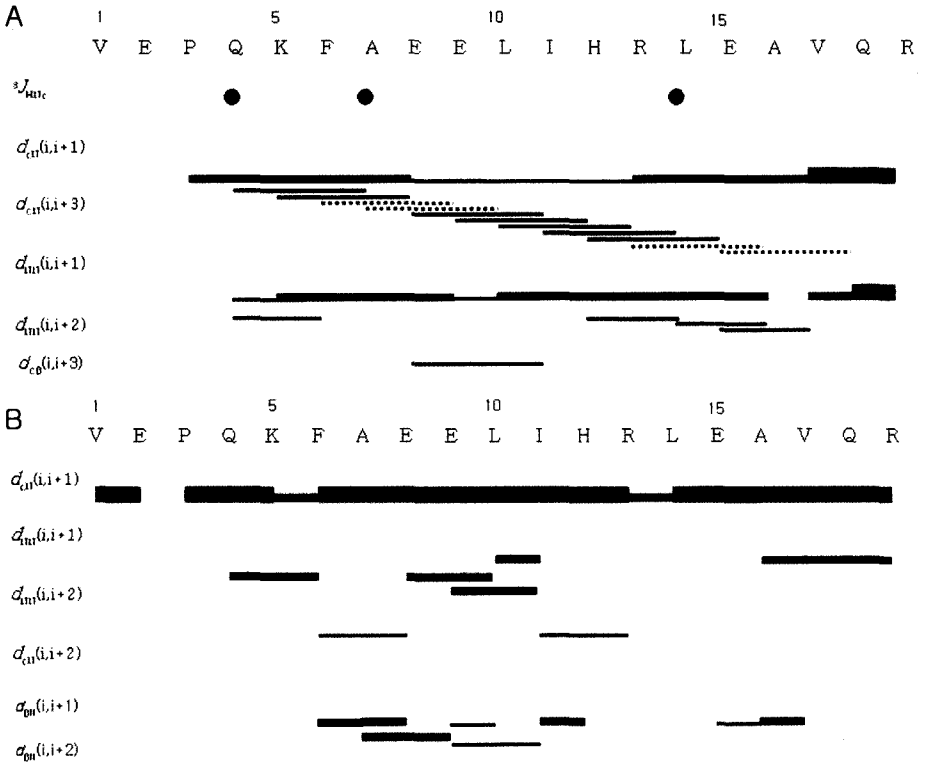

Fig. 3. Summary of NMR data for $axin^{pep}$ in (A) 70% H_2O/30% TFE solution at pH 7.0, 25℃ and (B) 90% H_2O/10% ^2H_2O solution at pH 7.0, 5℃ showing the NOE contacts and backbone vicinal coupling constants (●; $^3J_{HN\alpha}$< 6Hz)

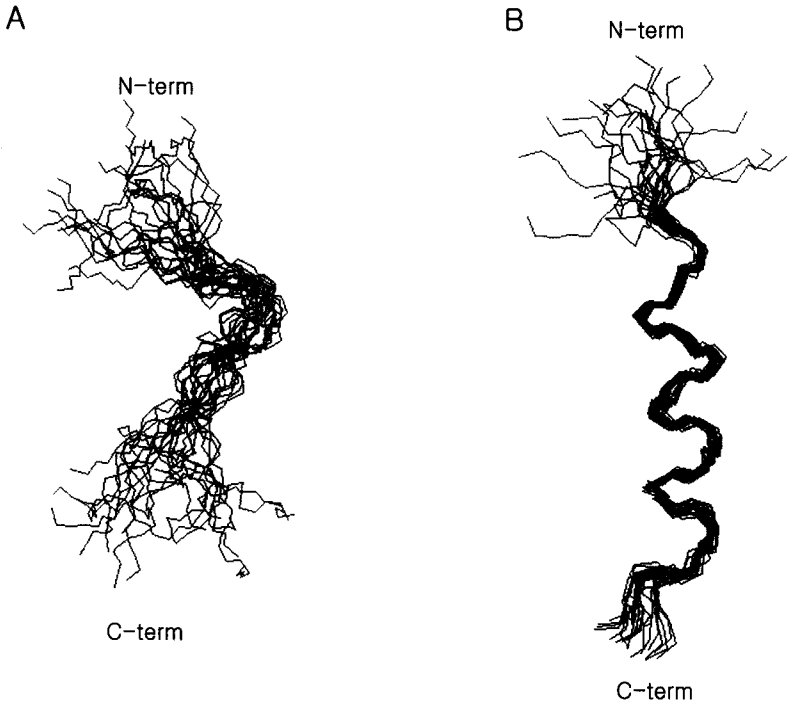

Fig. 4. Twenty susperimposed $<SA>_k$ structures of $axin^{pep}$ for H_2O solution (A) and TFE/H_2O solution (B), respectively.

Solution Structure

The NMR structures were calculated using the experimental restraints derived from 2D-NOESY and DQF-COSY spectra. A total of 50 distance geometry structures were served as starting structures for dynamical simulated-annealing calculations both in H_2O and TFE solutions. All of the structures showed no constraint violations greater than 0.5 Å for distances and 3° for torsion angles. The 20 lowest energy structures ($<SA>_k$) of 50 simulated-annealing structures were selected for detailed structural analysis. The average structure ($<SA>_k$) was calculated from the geometrical average from 20 $<SA>_k$ structures coordinates and subjected to restraint energy minimization (REM) to correct covalent bonds and angle distortions. The energies and structural statistics of the final 20 simulated-annealing structures related to experimental constraints were listed in the Table 1. In the TFE environment, the atomic rmsds of the final 20 structures for the individual residues

Table 1. Structural statistics for the final simulated-annealing structures of axin^{pep} peptide

	H_2O		TFE	
	$<SA>_k$	$<\overline{SA}>_{kr}$	$<SA>_k$	$<\overline{SA}>_{kr}$
Energy				
Total	48.97	32.57	76.70	43.37
Bonds	3.05	1.79	6.17	3.26
Angles	18.74	11.42	26.97	17.51
Improper	0.50	0.29	1.57	0.41
Van der Waals	9.78	5.75	11.70	0.21
NOE	16.90	9.06	30.28	21.06
Lennard-Jones†	-31.05	-26.49	-51.27	-50.30
RMSD				
Bonds(Å)	0.0030	0.0020	0.0043	0.0031
Angles(deg)	0.4520	0.4238	0.5431	0.4377
NOE(Å)	0.0340	0.0331	0.0381	0.0390

†Lennard-Jones/Van der Waals potential was calculated using the CHARMm empirical energy function.

were calculated with respect to the average structure. The rmsds for the 20 structures were 0.086 nm for backbone atoms and 0.195 nm for all heavy atoms, respectively. The result in the Ramachandran plot indicted that the φ, ψ angles of the 20 final structures are properly distributed in energetically acceptable regions. A best-fit superposition of the 20 final NMR structures is displayed in Fig. 4. Axin^{pep} in aqueous solution (Fig. 4A) shows a nascent helix[19] spanning residues from Glu^{390} to Ile^{393}.

The axin^{pep} in TFE/water mixture (Fig. 4B) becomes a standard α-helical conformation for residues from Gln^{391} to Val^{399}. It has been reported that C-terminal hydrophobic residues of GSK3β make hydrophobic interaction with central residues of the axin^{pep} peptide,

providing that the helical structure of $axin^{pep}$ is important in Wnt signalling.

Acknowledgements

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