Available online at www.sciencedirect.com
ScienceDirect
Biochemical and Biophysical Research Communications 350 (2006) 120–124
BBRC
www.elsevier.com/locate/ybbrc

Conformational analysis in solution of gastrin releasing peptide

Choonshik Shin [a], K. Hun Mok [b], Jin Hee Han [a], Joong-Hoon Ahn [a], Yoongho Lim [a,*]

[a] *Bio/Molecular Informatics Center, Division of Bioscience and Biotechnology, IBST, Konkuk University, Seoul 143-701, Republic of Korea*
[b] *Department of Chemistry and Oxford Centre for Molecular Sciences, University of Oxford, Oxford OX1 3QZ, UK*

Received 29 August 2006
Available online 18 September 2006

Abstract

Gastrin releasing peptide (GRP) is the first peptide isolated from porcine gastric and intestinal tissues and is homologous to the carboxyl terminus of bombesin (Bn) isolated from the skin of the frog *Bombina bombina*. It is a member of the Bn-like peptides, which are important in numerous biological and pathological processes. The Bn-like peptides show high sequence homology in their C-terminal regions, but they have different selectivity for their receptors. In particular, GRP selectively binds to the GRP receptor (GRPR). However, the molecular basis for this selectivity remains largely unknown. Here, we report the three-dimensional structure of GRP. Hopefully, it could be helpful in a better understanding of the binding selectivity between GRP and GRPR.

Keywords: NMR; Gastrin releasing peptide; Three-dimensional structure; Bombesin-like peptides; Gastrin releasing peptide receptor

Gastrin releasing peptide (GRP) is a 27-residue peptide that belongs to the family of bombesin (Bn)-like peptides and was originally isolated from porcine stomach by using the release of gastrin as a bioassay [1,2]. The Bn-like peptides were first characterized in frog skin and later found to have a broad distribution and a wide of actions in mammals, including involvement in the secretion of gastrointestinal peptide hormones [3], regulation of smooth muscle contraction and body temperature [4,5], and functioning as autocrine growth factors in human small-cell lung cancer [6]. In mammals, GRP and neuromedin B (NMB) are typical Bn-like peptides. Despite sharing a high sequence homology in their C-terminal regions (Table 1), Bn-like peptides exhibit different selectivities for the Bn receptors [7]. The Bn receptor family includes the GRP receptor (GRPR) to which GRP selectively binds, the neuromedin B receptor (NMBR), the amphibian bombesin receptor subtype 4 (BB4), and the orphan bombesin receptor subtype 3 (BRS-3). It is known that GRP has a few hundred-fold and a thousand-fold higher affinity for the GRPR than NMBR and BRS-3, respectively [8,9]. Recent mutational and molecular modeling studies of GRPR suggest the importance of several key amino acids in the GRPR for GRP selectivity [8]. For instance, residues such as K101, A198, S292, and T294 in GRPR and BRS-3 are likely to contribute to different selectivities for GRP [8]. Together with the studies on receptors, structural studies on Bn-like peptides have also been reported with interestingly somewhat diverging results. For example, bombesin exhibited an extended flexible structure in water or lysolecithin micelles [10,11], while NMB adopted a relaxed helical conformation in aqueous TFE solution and SDS micelles [12]. In order to find out whether significantly higher affinity of GRPR for GRP originated from the N-terminal peptide sequence or from an increased presence of secondary structure, we determined the three-dimensional structure of GRP using NMR spectroscopy and molecular dynamics simulations.

Materials and methods

Sample preparation. Gastrin releasing peptide (GRP) was synthesized by American Peptides Co. Inc. (Sunnyvale, CA, USA). Its primary structure is Val^{1}-Pro-Leu-Pro-Ala-Gly-Gly-Gly-Thr-Val-Leu-Thr-Lys-Met-Tyr-Pro-Arg-Gly-Asn-His-Trp-Ala-Val-Gly-His-Leu-Met^{27}, and its

* Corresponding author. Fax: +82 2 453 3761.
E-mail address: yoongho@konkuk.ac.kr (Y. Lim).

0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.09.022

Table 1
Comparison of the primary structure of GRP with those of other bombesin-like peptides

Peptide	Sequence
Gastrin releasing peptide (GRP)	VPLPAGGGTVLTKMYPR**GNHWAVGHLM-NH$_2$**
Neuromedin B (NMB)	**GNLWATGHFM-NH$_2$**
Bombesin (Bn)	EQRL**GNQWAVGHLM-NH$_2$**

Bold letters denote identical residues.

molecular weight is 2859.3 Da. The mass data obtained by ESI-MS (VG platform, VG BIOTECH, Manchester, UK) were m/z 1430.5 ($[M+2H]^{2+}$) and the purity of the peptide determined by reversed-phase HPLC (Waters, Milford, MA, USA) was 98%. For HPLC analysis, the following conditions were used: an injection volume, 20 μl; column, C18 silica (4.6 × 250 mm); eluent A, 0.1% trifluoroacetic acid (TFA) in water; eluent B, 0.1% TFA in acetonitrile; flow rate, 1.5 ml/min; detection wavelength, 215 nm.

NMR spectroscopy. All NMR measurements were performed on a Bruker Avance 600 spectrometer system (14.1 T, Karlsruhe, Germany) with a cryoprobe at 298 K. The NMR spectra of ^{1}H NMR, correlated spectroscopy (COSY), total correlated spectroscopy (TOCSY), and nuclear Overhauser and exchange spectroscopy (NOESY) were collected in 90% H_2O/10% D_2O. The concentration of the sample was approximately 1 mM. For ^{1}H NMR analysis, 16 transients were acquired with a 1 sec relaxation delay using 32 K data points. The 90° pulse was 11.0 μs with a spectral width of 9615 Hz. Two-dimensional spectra were acquired with 2048 data points for t2 and 256 for t1 using time proportional phase increments except the COSY experiment, where magnitude mode was applied. The NOESY experiment was performed with a mixing time of 400 ms. The mixing time for TOCSY with MLEV17 spin-lock pulse program was 80 ms. In all experiments, water peaks were suppressed by presaturation. Prior to Fourier transformation, zero filling of 2 K and sine squared bell window function were applied using XWIN-NMR (Bruker, Karlsruhe, Germany) [13].

Structure calculation. Data analysis was carried out using Sparky [14]. Molecular modeling calculations, graphical representation, and structural analyses were carried out with Insight II (Accelrys, San Diego, CA, USA) on an O2 R12,000 Silicon Graphics workstation [15]. Distance constraints were calculated using the relationship $\eta_{ij}/\eta_{kl} = (r_{kl}/r_{ij})^6$, where η and r are the nOe intensity and distance for the hydrogen atom pairs i, j and k, l, respectively [16]. The interproton distance between geminal amide protons of the Asn[19] side chain was used as a reference distance of 1.74 Å. nOe intensities were classified as strong, medium, and weak based on their distance ranges of 1.8–2.5, 2.5–3.5 and 3.5–5.0 Å, respectively. Dihedral angles were obtained from the Karplus equation, $^3J_{NH\alpha} = 6.7\cos^2\phi - 1.3\cos\phi + 1.5$, where ϕ is the dihedral angle between the NH and CαH proton [17]. The force field used for molecular dynamics (MD) and energy minimization was the consistent valence force field (cvff) provided by Accelrys. Energy minimization of the structures included steepest descents carried out until a maximum derivative of 0.1 kcal/molÅ, and conjugate gradients followed until a maximum derivative of 0.01 kcal/molÅ. After energy minimization, MD was performed from 300 to 1000 K with 100 K increment steps. At every step, MD was carried out for 2 ps. At 1000 K, MD was performed for

Table 2
^{1}H chemical shifts (δ, ppm) of GRP in water at 298 K

Residue	Chemical shift				$^3J_{NH\alpha}$ (Hz)
	NH	CαH	CβH	CγH and others	
Val[1]	—	4.08	2.21	0.90, 1.01	—[a]
Pro[2]	—	4.32	2.19, 1.81	1.91; CδH 3.76, 3.53	7.4
Leu[3]	8.34	4.50	1.49	1.66; CδH 0.87, 0.85	6.2
Pro[4]	—	4.41	2.21, 1.78	1.89; CδH 3.65, 3.53	—
Ala[5]	8.39	4.21	1.31	—	10.2
Gly[6]	8.38	3.89	—	—	—
Gly[7]	8.26	3.90	—	—	—
Gly[8]	8.29	3.92	—	—	—
Thr[9]	8.06	4.25	4.08	1.08	7.8
Val[10]	8.18	4.01	1.96	0.84	7.3
Leu[11]	8.32	4.33	1.56	1.53; CδH 0.85, 0.78	6.8
Thr[12]	7.97	4.16	4.08	1.09	—
Lys[13]	8.14	4.15	1.62, 1.57	1.23; CδH 1.27; CεH 2.85	7.2
Met[14]	8.08	4.32	1.79	2.39, 2.30	—
Tyr[15]	8.22	4.47	2.48	CδH 7.00; CεH 6.73	—
Pro[16]	—	4.30	2.13, 1.79	1.86; CδH 3.62, 3.45	—
Arg[17]	8.34	4.23	1.77, 1.65	1.53; CδH 3.02; εNH 7.04, 6.70	—
Gly[18]	8.28	3.83	—	—	—
Asn[19]	8.14	—	2.93, 2.72	γNH_2 7.37, 6.76	7.2
His[20]	8.34	4.50	3.00, 2.92	2H 8.36; 4H 6.96	—
Trp[21]	7.95	4.53	3.14, 3.06	2H 7.09; 4H 7.43; 5H 7.02; 6H 7.12; 7H 7.36; εNH 10.01	5.8
Ala[22]	8.07	4.18	1.18	—	—
Val[23]	7.94	3.91	1.94	0.84	6.1
Gly[24]	8.36	3.81	—	—	—
His[25]	8.21	4.54	3.11, 3.01	2H 8.41; 4H 7.07	—
Leu[26]	8.27	4.21	1.53	1.46; CδH 0.81, 0.75	—
Met[27]	8.30	4.33	1.97, 1.87	2.49, 2.39	—

[a] Not observed.

20 ps, and the output conformers were collected at every 0.2 ps and 100 conformers were saved in the history file. The conformer with the lowest total energy was chosen and used for further MD simulation from 1000 to 300 K with 100 K steps. At 300 K, MD was carried out for 20 ps with 1 fs steps. Its history file was written every 0.2 ps. Among the 100 conformers, 10 conformers showing the lowest total energy were selected and superimposed. Of these, the conformer with the lowest energy was chosen and a statistical evaluation was performed using PROCHECK [18].

Results and discussion

The NMR data obtained in aqueous solution (90% H_2O/10% D_2O) were assigned as follows. Among the 27 residues and excluding the N-terminal Val[1] and the three Pro side chains (Pro[2], Pro[4], and Pro[16]), 22 of the 23 possible cross peaks between NH and CαH were observed in the COSY spectrum (Supplementary Fig. 1). However, the Pro residues were easily assigned based on the TOCSY spectrum (Supplementary Fig. 2). The remaining Asn[19] did not show a cross peak between NH and CαH by virtue of its proximity to the water peak; however, a peak between NH and CβH was observable. Moreover, the connectivities among NH, CαH, CβH, CγH, and other protons and their chemical shift assignments were based on the TOCSY and COSY spectra (Supplementary Figs. 2 and 3). The γNH_2 protons of Asn[19] were assigned to be 6.76 and 7.37 ppm. The aromatic protons of Tyr[15], His[20], Trp[21], and His[25] were assigned (Supplementary Fig. 4). The resulting ^1H chemical shifts of the GRP are listed in Table 2.

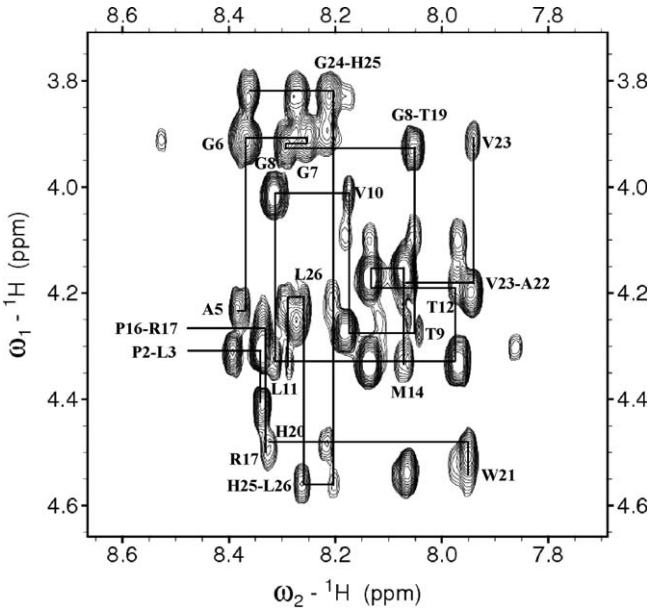

Fig. 1. The NOESY spectrum showing $C\alpha H_i$–NH_{i+1}cross peaks. The sequential assignments are indicated by solid lines.

A sequential assignment was obtained from the NOESY spectrum as shown in the fingerprint (Fig. 1) and the NH–NH regions (Supplementary Fig. 5). A total of 111 nOe cross peaks observed. Among them, 54 were inter-residue and sequential nOes. The sequential and medium-range nOes, $^3J_{NH\alpha}$ coupling constants, and the chemical shift indices (CSI) [19] are summarized in Fig. 2. While a continuous negative value from the chemical shift index indicates that residues Thr[12] to Gly[18] may be involved in helical structure, the spectral information does not provide any clear evidence for significant, well-defined secondary structure elements.

In the simulated annealing, 10 structures of low total energy were obtained using the distances calculated from nOe data and the dihedral angles from $^3J_{NH\alpha}$. In the final simulated annealing step, the peptide was subjected to energy minimization and molecular dynamics at 300 K, 1 atm for 20 ps with 1 fs at each step (Supplementary Fig. 6). The total energy was ranged within 10 kcal/mol. In addition, the value of the root mean square deviation (RMSD) of 10 superimposed backbone structures was 0.91 Å (Supplementary Fig. 7). Therefore, it could be considered that the structure of GRP was conserved during MD simulations. The structure with the lowest total energy was chosen and evaluated using PROCHECK. The statistical analysis of Ramachandran plot showed that 47.2% of the residues are in the most favored region, 52.8% in the additional allowed region, and 0% in the generously allowed region and the disallowed region (Supplementary Fig. 8). Summary of experimental constraints and statistical analysis results are listed in Table 3.

As mentioned before, we expected the peptide may have a helix based on its chemical shift index (CSI) analysis. However, as shown in Fig. 3, there is not a helix in the peptide structure between Thr[12] and Gly[18]. This result was not consistent with those obtained from the CSI. On

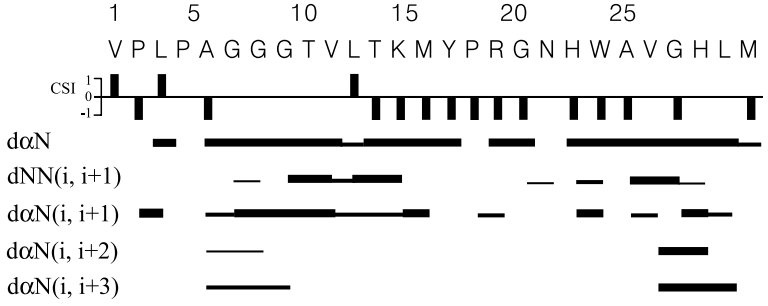

Fig. 2. The nOe sequential map and chemical shift indices (CSI) of GRP.

Table 3
Summary of experimental constraints and statistical analysis of GRP

Experimental distance constraints	
Total number of nOe	111
Intra-residue nOe[a]	57
Inter-residue nOe	26
Sequential nOe	28
Dihedral constraints	10
RMSD (Å) to a mean structure	
Backbone atoms	0.91 ± 0.23
Heavy atoms	0.97 ± 0.25
Ramachandran plot of residues[b](%)	
In most favored regions	47.2
In additional allowed regions	52.8
In generously allowed regions	0
In disallowed regions	0

[a] Not used in the structure calculation.
[b] Calculated by PROCHECK.

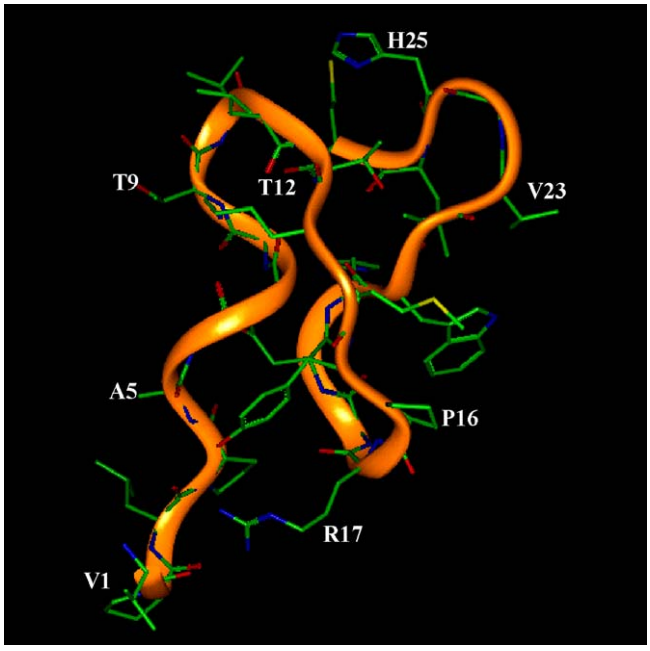

Fig. 3. Ribbon representation of the overall NMR structure of GRP.

the other hand, interestingly, the N-terminal region of the peptide between Ala[5] and Thr[9] showed a short helix-like structure. Furthermore, the Bn-like peptides share the similar amino acids in their C-terminal regions (Table 1) and the amino acids are considered to play an important role in selective binding for the Bn receptors. In addition, the C-terminal structure of GRP is similar to that of bombesin. They show flexible conformations in water or lysolecithin micelles [10,11]. In the case of NMB, it has an ordered helical structure in aqueous TFE solution and SDS micelles [12].

The Connolly surface of GRP was evaluated by Insight II, which provided rich information regarding the hydrophobicity of the peptide. The peptide consists of fifteen hydrophobic residues and seven hydrophilic residues. As expected, its overall property of the surface is hydrophobic as shown in Fig. 4.

In conclusion, using NMR and MD simulations we have obtained the solution structure of GRP. The overall structure of the peptide did not show any ordered secondary structure, but in the N-terminal region of the peptide, a shot helix-like structure was observed. In order to understand the structural basis for the selectivity of GRP for the GRPR, not only the structure of GRP but also that of GRPR is needed. Therefore, we can only conjecture the counter site of GRPR for binding based on our result. As shown in Fig. 4, GRP has large hydrophobic surface composed of V10, V23, L3, and M14, and small hydrophilic surface of R17. Accordingly, the binding site of GRPR for GRP could consist of large hydrophobic and small hydrophilic surface. Nakagawa et al. [8] suggested the putative binding site of GRPR obtained by molecular dynamics, where 8 residues within 6Å of the proposed binding pocket such as H186, V187, K188, D189, A198, S293, E294, and T304 of GRPR are the key residues for GRP selectivity. However, it was not consistent with our conjecture. Unfortunately, any 3D structures of bombesin receptor family, including GRPR and bombesin-like peptides have not been solved yet. Because GRPR is a membrane protein and the structural study on the GRPR is

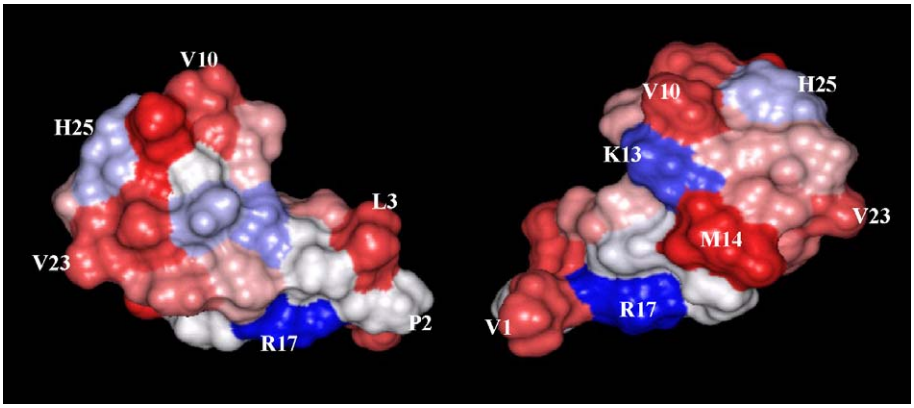

Fig. 4. Two different side-views of the Connolly surface of GRP. (red: the most hydrophobic; blue: the most hydrophilic; white: medium). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

relatively difficult to carry out with other cytosolic proteins, the structure of GRP obtained from this experiment cannot be compared with them. However, we hope that its structure will be solved in the near future, and our structural studies on GRP could be helpful in a better understanding of the binding selectivity between GRP and GRPR.

Acknowledgments

This work was supported by Biogreen 21 (Korea Ministry of Agriculture and Forestry) and the second Brain Korea 21 (Korea Ministry of Education) grants.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.09.022.

References

[1] E.R. Spindel, W.W. Chin, J. Price, L.H. Rees, G.M. Besser, J.F. Habener, Cloning and characterization of cDNAs encoding human gastrin-releasing peptide, Proc. Natl. Acad. Sci. USA 81 (1984) 5699–5703.

[2] T.J. McDonald, H. Jörnvall, G. Nilsson, M. Vagne, M. Ghatei, S.R. Bloom, V. Mutt, Characterization of a gastrin releasing peptide from porcine non-antral gastric tissue, Biochem. Biophys. Res. Commun. 90 (1979) 227–233.

[3] M.A. Ghatei, R.T. Jung, J.C. Stevenson, C.J. Hillyard, T.E. Adrian, Y.C. Lee, N.D. Christofides, D.L. Sarson, K. Mashiter, I. MacIntyre, S.R. Bloom, Bombesin: action on gut hormones and calcium in man, J. Clin. Endocrinol. Metab. 54 (1982) 980–985.

[4] V. Erspamer, Discovery, isolation, and characterization of bombesin-like peptides, Ann. N.Y. Acad. Sci. 547 (1988) 3–9.

[5] M.R. Brown, K. Carver, L.A. Fisher, Bombesin: central nervous system actions to affect the autonomic nervous system, Ann. N.Y. Acad. Sci. 547 (1988) 174–182.

[6] F. Cuttitta, D.N. Carney, J. Mulshine, T.W. Moody, J. Fedorko, A. Fischler, J.D. Minna, Bombesin-like peptides can function as autocrine growth factors in human small-cell lung cancer, Nature 316 (1985) 823–826.

[7] S.A. Mantey, H.C. Weber, E. Sainz, M. Akeson, R.R. Ryan, T.K. Pradhan, R.P. Searles, E.R. Spindel, J.F. Battey, D.H. Coy, R.T. Jensen, Discovery of a high affinity radioligand for the human orphan receptor, Bombesin receptor subtype 3, J. Biol. Chem. 272 (1997) 26062–26071.

[8] T. Nakagawa, S.J. Hocart, M. Schumann, J.A. Tapia, S.A. Mantey, D.H. Coy, K. Tokita, T. Katsuno, T. Jensen, Identification key amino acids in the GRPR responsible for high affinity binding of GRP, Biochem. Pharmacol. 69 (2005) 579–593.

[9] K. Ttokita, S.J. Hocart, D.H. Coy, R.T. Jensen, Molecular basis of the selectivity of gastrin-releasing peptide receptor for gastrin-releasing peptide, Mol. Pharmacol. 61 (2002) 1435–1443.

[10] M.D. Díaz, M. Fioroni, K. Burger, S. Berger, Evidence of complete hydrophobic coating of bombesin by trifluoroethanol in aqueous solution, Chem. Eur. J. 8 (2002) 1663–1669.

[11] P. Cavatorta, A. Spisni, A.G. Szabo, G. Farruggia, L. franzoni, L. Masotti, Conformation of bombesin in buffer and in the presence of lysolecithin micelles: Nmr, CD, and fluorescence studies, Biopolymers 28 (1989) 441–463.

[12] S. Lee, Y. Kim, Solution structure of neuromedin B by ^{1}H nuclear magnetic resonance spectroscopy, FEBS Lett. 460 (1999) 263–269.

[13] H. Yang, K. Kang, Y. Lim, NMR study of a novel pentasaccharide isolated from *Penicillium citrium*, Magn. Reson. Chem. 41 (2003) 223–226.

[14] T.D. Goddard, D.G. Kneller, Sparky Program for NMR Assignment and Integration, Computer Graphics Laboratory, University of California, San Francisco.

[15] C. Shin, J.H. Ahn, Y. Lim, Conformational analysis in solution of protein kinase C βII V5-1 peptide, Biochem. Biophys. Res. Commun. 337 (2005) 154–159.

[16] K. Wüthrich, NMR of Proteins and Nucleic Acids, John Wiley & Sons, New York, 1986.

[17] A. Pardi, M. Billeter, K. Wüthrich, Calibration of the angular dependence of the amide proton-C_α proton coupling constants, $^{3}J_{HN\alpha}$, in a globular protein, J. Mol. Biol. 180 (1984) 741–751.

[18] R.A. Laskowski, M.W. MacArthur, D.S. Moss, J.M. Thornton, PROCHECK: a program to check the stereochemical quality of protein structures, J. Appl. Cryst. 26 (1993) 283–291.

[19] D.S. Wishart, B.D. Sykes, F.M. Richards, The chemical shift index: a fast and simple method for the assessment of protein secondary structure through NMR spectroscopy, Biochemistry 31 (1992) 1647–1651.