Journal of the Korean Magnetic Resonance Society **2007**, *11*, 85 - 94

Structural Study of the Cytosolic C-terminus of Vanilloid Receptor 1

Min-Duk Seo[1], Hyung-Sik Won[2], Uhtaek Oh[3] and Bong-Jin Lee[1*]

[1]Research Institute of Pharmaceutical Sciences, College of Pharmacy, Seoul National University, Seoul 151-742, Korea
[2]Department of Biotechnology, Division of Life Sciences, College of Biomedical & Health Science, Konkuk University, Chungju, Chungbuk 380-701, Korea
[3]Sensory Research Center, CRI, Seoul National University, Seoul 151-742, Korea
Received April 25, 2007

Abstract : Vanilloid receptor 1 [transient receptor potential vanilloid subfamily member 1 (TRPV1), also known as VR1] is a non-selective cationic channel activated by noxious heat, vanilloids, and acid, thereby causing pain. VR1 possesses six transmembrane domain and N- and C-terminus cytosolic domains, and appears to be a homotetramer. We studied the structural properties of C-terminus of VR1 (VR1C) using CD and NMR spectroscopy. DPC micelles, with a zwitterionic surface, and SDS micelles, with a negatively charged surface, were used as a membrane mimetic model system. Both SDS and DPC micelles could increase the stability of helical structures and/or reduce the aggregation form of the VR1C. However, the structural changing mode of the VR1C induced by the SDS and DPC micelles was different. The changes according to the various pHs were also different in two micelles conditions. Because the net charges of the SDS and DPC micelles are negative and neutral, respectively, we anticipate that this difference might affect the structure of the VR1C by electrostatic interaction between the surface of the VR1C and phospholipids of the detergent micelles. Based on these similarity and dissimilarity of changing aspects of the VR1C, it is supposed that the VR1C probably has the real pI value near the pH 7. Generally, mild extracellular acidic pH (6.5~6.8) potentiates VR1 channel activation by noxious heat and vanilloids, whereas acidic conditions directly activate the channel. The channel activation of the VR1 might be related to the structural change of VR1C caused by pH (electrostatic interactions), especially near the pH 7. By measuring the ^{1}H-^{15}N TROSY spectra of the VR1C, we could get more resolved and dispersed spectra at the low pH and/or detergent micelles conditions. We will try to do further NMR experiments in low pH with micelles conditions in order to get more information about the structure of VR1C.
Keywords: C-terminus of Vanilloid receptor 1 (VR1C), CD spectroscopy, NMR spectroscopy, detergent micelles, electrostatic interaction

*To whom correspondence should be addressed. E-mail : lbj@nmr.snu.ac.kr

INTRODUCTION

The vanilloid receptor 1 [transient receptor potential vanilloid subfamily member 1 (TRPV1), also known as VR1] is a ligand-gated cationic channel activated by vanilloids, acid, and heat, thereby causing pain.[1-3] Noxious thermal stimuli (over 43℃), protons (acidic pH), or capsaicin are required to open the VR1 cahnnel.[4,5] VR1 belongs to a class of transient receptor potential channels possessing six transmembrane domains and N- and C-terminus cytosolic domains(Fig. 1).[3,6] There is a stretch linking the fifth and sixth segments that contains an amphipathic fragment denoted as the P-loop, which contributes to its permeation properties.[7] The cytosolic C-terminus contains a Walker A type ATP binding site, calmodulin binding site, phosphorylation site for protein kinase C and phosphatydylinositol-4,5-biphosphate binding sites.[8-11] Structurally, VR1 appears to be a tetramer formed by the assembly of four identical subunits around a central aqueous pore,[12,13] and TRP-like domain (comprising ^{684}Glu–^{721}Arg) in the C-terminus of VR1 acts as an association domain (AD) of the protein.[14]

Recently, it was suggested that an increased VR1 expression on myelinated fibres might contribute to the anti-hyperalgesic effect of topical capsaicin in diabetic neuropathic pain.[15] This consideration would also apply to VR1 antagonists. Actually, there are considerable investments to discover the potent and selective small-molecule VR1 antagonists, some of which are already undergoing clinical trials for the indications of chronic inflammatory pain and migraine.[16]

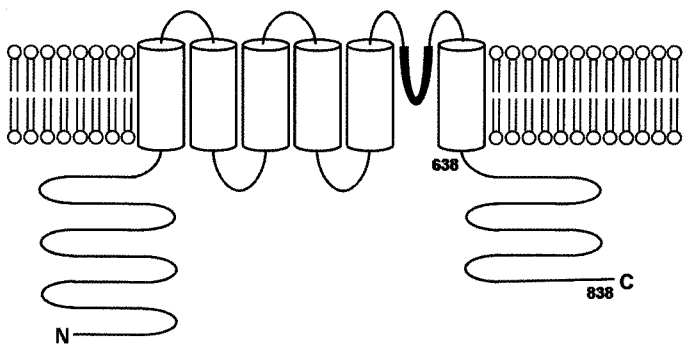

Fig. 1. The topology of the Vanilloid receptor 1 (VR1) in the membrane. The residues number of the C-terminus of VR1 (VR1C; from residue 638 to 838) is labeled.

Here, we characterize the structural properties of C-terminus of vanilloid receptor 1 (VR1C hereafter) using CD and NMR spectroscopy.

EXPERIMENTAL

Protein expression and purification

The gene which encoding C-terminal domain of vanilloid receptor 1 (VR1C; residues 683-838) was cloned into the expression vector pET-28a and was expressed in the *Escherichia coli* BL21 (DE3) strain. Cells were grown in LB medium containing 50ug/ml kanamycin at 37℃ until an OD_{600} of 0.6 and then induced with 0.5 mM IPTG for 4hr. The cell pelletes were lysed and disrupted by sonication with 50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl. Since overexpressed VR1C was insoluble, the proteins could be obtained by the refolding process. The pellet was washed with 1 M urea buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1M urea), and the washed supernatant was discarded. Then, the washed pellet was resuspened with 8 M urea solubilization buffer, and the solution was centrifuged. The supernatant was applied onto Ni^{2+} affinity column (Chelating Sepharose Fast Flow, Pharmacia) equilibrated with 8 M urea solubilization buffer. The protein was eluted by the linear gradient with the same buffer containing from 5 mM to 300 mM imidazole. The eluted VR1C was dialyzed at 4℃ against 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM beta-mercaptoethanol in the presence of 4 M urea. The protein was dialyzed against 2 M urea, 1M urea, and same buffer without urea (step by step). The purified VR1C was concentrated using Centriprep and Centricon (Amicon).

CD (Circular Dichroism) spectroscopy

CD spectra were obtained at 20℃ on a JASCO J-720 spectropolarimeter using a 0.2 cm path length cell. CD scans were taken at 293 K from 250 to 190 nm, with a 1 nm bandwidth, a 4s response time, a scan speed of 50 nm/min. Three scans were added and averaged, followed by subtraction of the CD signal of the solvent. To investigate the thermodynamic properties of VR1C, temperature scan was performed at 222 nm in a

temperature range between 20℃ and 90℃. The concentration of VR1C was 10 uM, and the concentrations of SDS and DPC in pH titration experiment were 10mM and 4 mM, respectively.

NMR spectroscopy

^{15}N-labeled VR1C was prepared from a culture grown in M9 media containing [^{15}N]NH4Cl. Samples for NMR measurements contained 0.5 mM VR1C dissolved in 90% H_2O/10% D_2O, containing 25 mM potassium phosphate, 150 mM NaCl, and various pHs (pH 7, 5, and 4). NMR experiments were performed at 303K or 313K on a Bruker DRX 500 spectrometer. The NMR data were processed by NMRPipe[17] and analyzed with the NMRView.[18]

RESULTS AND DISCUSSION

Effects of detergent micelles on the structure of the VR1C

The far-UV CD spectra were measured to analyze the secondary structure of the VR1C. DPC micelles, with a zwitterionic surface, and SDS micelles, with a negatively charged surface, were used as model system that resemble the environment of a biological phospholipid bilayer.[19,20] The CD curves reveal that the structure of the VR1C is not so highly ordered in the aqueous solution, but adopts stable α -helical structure in the presence of micelles (Fig. 2 A and B). However, the structural changing mode of the VR1C due to the SDS micelles and DPC micelles is different. In the case of the SDS micelles, 1 mM SDS was sufficient to saturate the secondary structure of the VR1C, that is to say, CD curves of the VR1C was not changed anymore by adding the more concentrated SDS (final concentration of SDS was 100 mM). But, DPC micelles could change the CD spectra of the VR1C gradually up to 20 mM DPC. This suggests that the structure of the VR1C is sensitive to the charge of the membrane as well as the membrane mimetic environment itself. Since the net charges of the SDS and DPC micelles are negative and neutral, respectively, this difference might affect the structure of the VR1C by electrostatic interaction between the surface of the VR1C and phospholipids of the

detergent micelles. To know the more detailed effects of the electrostatic interactions on the VR1C structure, we performed CD and NMR spectroscopy at various pHs.

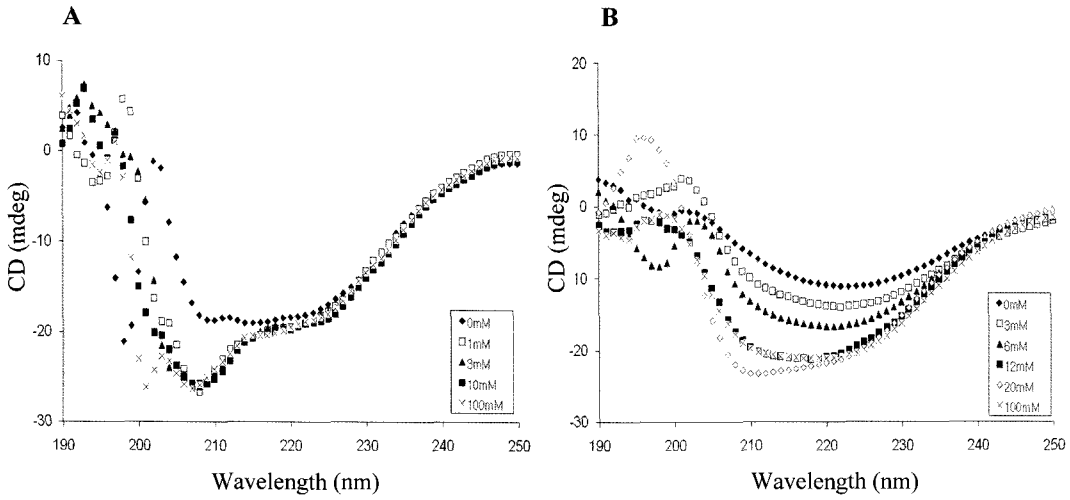

Fig. 2. Far-UV CD spectra of the C-terminus of Vanilloid receptor 1 (VR1C). (A) Structural changes of VR1C against the various concentration (from 0 mM to 100 mM) of SDS (A) and DPC micelles (B).

Effects of pH on the structure and function of the VR1C

In order to understand the VR1C behavior as a function of pH, CD spectra of the VR1C were measured with a range between pH 4 and pH 8 in the presence of SDS or DPC micelles. The effects of pH was also different according to a kind of micelles. As shown in Fig. 3A, in buffers with SDS micelles, the intensities of the CD curves of the VR1C was gradually increased as the pH was decreased from pH 8 (and pH 7) to pH 4, which means that the VR1C adopts an ordered structure in lower pH. However, CD patterns of the VR1C in DPC micelles (Fig. 3B) were different to those in SDS micelles. Although the intensity of the CD curve at pH 4 was the most strong, the other curves were not gradual. In contrast to the patterns in SDS micelles, the CD intensities at pH 7 and 8 in DPC micelles were more strong than pH 5, and the intensity at pH 6 was the most weak. In other words, at pH 7 and 8, the VR1C adopts less stable conformation than at pH 4, but more stable α -helical structure than at pH 5 and pH 6.

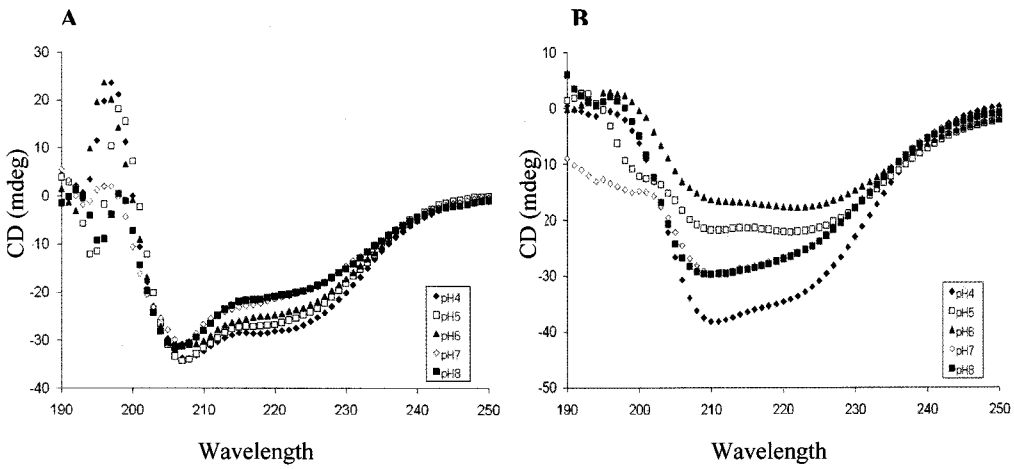

Fig. 3. Effect of pH on the structure of VR1C in the presence of 10 mM SDS (A), and 4 mM DPC (B).

It is noteworthy that both in SDS and DPC micelles, the CD pattern of the VR1C at pH 7 is identical to that of at pH 8. In SDS micelles, the CD values (mdeg) were decreased gradually from pH 4, and the decreasing CD values stopped at pH 7, and there were no more changes by increasing the pH (pH 8). In DPC micelles, the intensities of the CD curves were decreased from pH 4 to pH 6, gradually, but the CD values of the VR1C at pH 7 were rather increased, and the CD curve at pH 8 showed similar pattern to that at pH 7. Commonly, pH 7 might be a boundary (or standard) of the structural changes in both SDS and DPC micelles, although the changing patterns according to the pH was different to each other. Based on these similarity and dissimilarity of changing aspects of the VR1C, it is supposed that the VR1C probably has the real pI value near the pH 7. Generally, proteins adopt positively surface charges at the pHs which lower than the intrinsic pI, and vice versa. Therefore, at the low pHs (pH 4, 5, and 6), positively charged VR1C is stable in both SDS and DPC micelles by electrostatic interaction. However, when pHs are higher (pH 7 and 8), VR1C adopts negatively surface charge, so VR1C is more stable in DPC micelles with both positively and negatively surface charge than in SDS micelles with negatively charged surface.

Generally, mild extracellular acidic pH (6.5~6.8) potentiates VR1 channel activation by noxious heat and vanilloids, whereas acidic conditions directly activate the

channel.[4,5,14] The channel activation of the VR1 might be related to the structural change of VR1C caused by pH (electrostatic interactions), especially near the pH 7.

Optimal condition for NMR study

To understand the structural properties of the VR1C, we measured the ^{1}H-^{15}N TROSY spectra of the VR1C at various pHs. For the determination of the effect of pH, we prepared three kinds of the VR1C proteins which have different pHs (pH 7, 5, and 4). As shown in Fig. 4A and B, a few strong peaks (less than 30 peaks) appeared in TROSY spectra at pH 7, which suggesting that VR1C exists as the severe aggregation forms at pH 7. At the lower pH (pH 5 and pH 4), the spectra of the VR1C were more dispersed and many new peaks appeared (Fig. 4C and D). We also tested the micelles effects to enhance

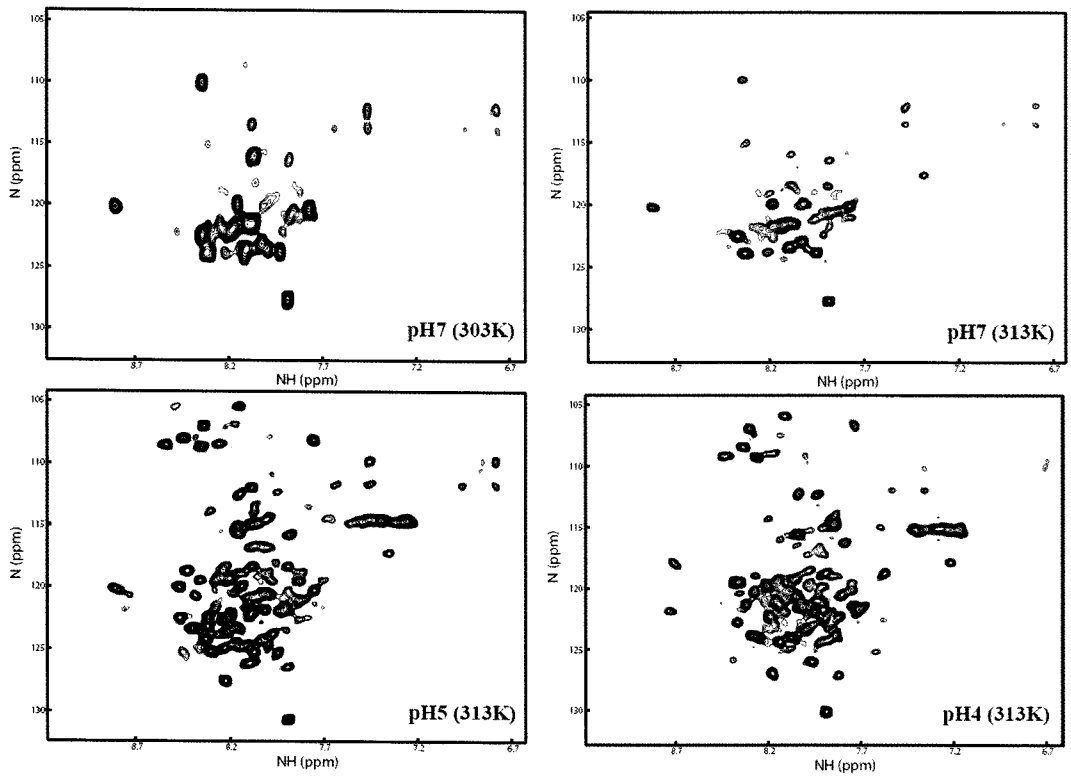

Fig. 4. ^{1}H-^{15}N TROSY spectra of the VR1C at various pH and temperature without micelles (A-D).

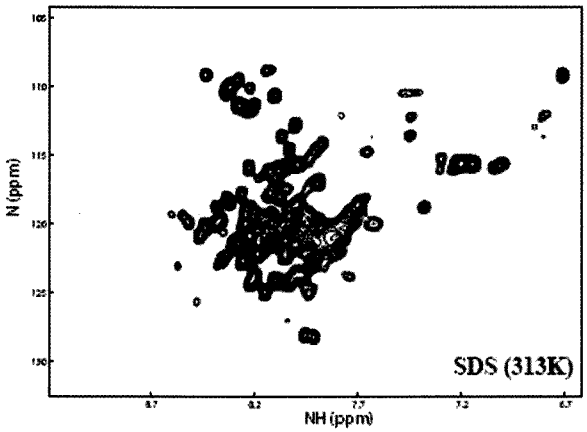

Fig. 5. ^{1}H-^{15}N TROSY spectra of the VR1C in SDS micelles buffer.

the quality of the spectra (Fig. 5). Compared to the low pH spectra, the overall resolution of the spectrum of the VR1C in SDS micelles did not improve significantly. We will try further NMR experiments in low pH with micelles condition, because the membrane mimetic environments can reflect the correct structural informations about membrane protein, VR1C.

Acknowledgments

This work is financially supported by the Ministry of Education and Human Resources Development (MOE), the Ministry of Commerce, Industry and Energy (MOCIE) and the Ministry of Labor (MOLAB) through the fostering project of the Lab of Excellency. This work was also supported by the Korea Science and Engineering Foundation(KOSEF) grant funded by the Korea government(MOST)(Innovative Drug Research Center for Metabolic and Inflammatory Disease) and 2007 BK21 project for Pharmacy.

REFERENCES

1. S. Bevan, J. Szocsanyi, *Trends Pharmacol. Sci.* **11**, 330-333(1990).
2. U. Oh, S.W. Hwang, D. Kim, *J. Neurosci.* **16**, 1659-1667(1996).

3. M.J. Caterina, M.A. Schumacher, M. Tominaga, T.A. Rosesn, J.D. Levine, D. Julius, *Nature* **389**, 816-824(1997).

4. M. Tominaga, M.J. Caterina, A.B. Malmberg, T.A. Rosen, H. Gilbert, K. Skinner, B.E. Raumann, A.I. Basbaum, D. J ulius, *Neuron* **21,** 531– 543(1998).

5. H.H. Chuang, E.D. Prescott, H. Kong, S. Shields, S.E. Jordt, A.I. Basbaum, M.V. Chao, D. Julius, *Nature* **411**, 957-962 (2001).

6. C. Harteneck, T.D. Plant, G.Schultz, *Trends Neurosci.* **23**, 159-166 (2000).

7. C. García-Martínez, C. Morenilla-Palao, R. Planells-Cases, JM. Merino, A. Ferrer-Montiel, *J Biol Chem.* **275**, 32552–32558 (2000).

8. J. Kwak, M.H. Wang, S.W. Hwang, T.Y. Kim, S.Y. Lee, U. Oh, *J. Neurosci.***20,** 8298– 8304 (2000).

9. M. Numazaki, T. Tominaga, H. Toyooka, M. Tominaga, *J. Biol.Chem.* **277,** 13375–13378 (2002).

10. M. Tominaga, T. Tominaga, Pflugers *Arch.-Eur. J. Physiol.* **451,** 143– 150 (2005).

11. A. Ferrer-Montiel, C. Garcıa-Martınez, C. Morenilla-Palao, N. Garcıa-Sanz, A. Fernandez-Carvajal, G. Fernandez-Ballester, R. Planells-Cases, *Eur. J. Biochem.* **271,** 1820– 1826 (2004).

12. N. Kedei, T. Szabo, J.D. Lile, J.J. Treanor, Z. Olah, M.J. Iadarola, P.M. Blumberg, *J. Biol. Chem.* **276**, 28613–28619 (2001).

13. Kuzhikandathil, E.V., Wang, H., Szabo, T., Morozova, N., Blumberg, P.M., Oxford, G.S. *J. Neurosci.* **21**, 8697–8706 (2001).

14. N. Garcia-Sanz, A. Fernandez-Carvajal, C. Morenilla-Palao, R. Planells-Cases, E. Fajardo-Sanchez, G. Fernandez-Ballester, A. Ferrer-Montiel, *J. Neurosci.* **24**, 5307-5314 (2004).

15. G. Lauria, M. Morbin, R. Lombardi, R. Capobianco, F. Camozzi, D. Pareyson, M. Manconi, P. Geppetti, *J. Periph. Nervous Syst.* **11**, 262– 271 (2006).

16. A. Szallasi, D.N. Cortright, C.A. Blum, S.R. Did, *Nat. Rev. Drug. Discov.* **6**, 357-372 (2007).

17. F. Delaglio, S. Grzesiek, G.W. Vuister, G. Zhu, J. Pfeifer, A. Bax, *J. Biomol. NMR* **6**, 277-293 (1995).

18. B.A. Johnson, R.A. Blevins, *J. Biomol. NMR* **4**, 603-614 (1994).

19. Z. Oren, Y. Shai, *Bioplymers* **47**, 451-463 (1998).

20. M.K. Yoon, S.H. Park, H.S. Won, D.S. Na, B.J. Lee, *FEBS Lett.* **484**, 241-245 (2000).