Journal of the Korean Magnetic Resonance Society **2006**, *10,* 155 - 162

Chmical Shift Variation of Bovine Angiogenin Upon Binding with Phosphate ions

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Received July 4, 2006

Abstract : Angiogenin is unique among angiogenic molecules in that it is a member of the pancreatic ribonuclease superfamily and, in fact, is a ribonucleolytic enzyme. Its enzymatic activity is extremely weak compared to that of the digestive RNases but is critical for its capacity to induce neovascularization. In this study, we completed the backbone resonance assignment of bovine angiogenin using triple resonance NMR experiments of ^{15}N and/or ^{13}C isotope labeled protein and investigated the chemical shift variation upon binding with inhibitor phosphate ion and determine the phosphate binding site.
Key words : angiogenesis, Angiogenin, protein structure

INTRODUCTION

The formation of new blood vessels is crucial for tissue regeneration and for efficient tumor growth, and requires the concerted action of various angiogenic factors and inhibitors. Angiogenesis is a complex process of new blood vessel formation, which is essential for cell reproduction, development and wound repair under normal condition. This process involves endothelial cell proliferation, migration, and membrane degradation.[1,2] Since angiogenesis is important for the growth and metastasis of tumors, many studies are being focused on the understanding of angiogenesis and inhibitors have received particular attention because of

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their therapeutic potential.[1-3] Now one of the angiogenic factors of angiogenesis is developing molecules, such as angiogenin.

Angiogenin (Ang) is a single-chain 14KDa protein implicated as an inducer of blood vessel formation. It was isolated originally from medium conditioned by human colon carcinoma cells in 1985 and subsequently from normal serum and milk. Angiogenin also is a member of the pancreatic ribonuclease superfamily.[4-8]

The well characterized human angiogenin (Ang; RNase 5) is an angiogenic factor involved in tumor progression.[9] Exhaustive structure and function relationship studies [10] have demonstrated that these activities could be circumscribed to, at least, three sites that are essential for angiogenic activity: a ribonucleolytic active site, [11] an endothelial cell binding site [12] and a nuclear localization site.[13]

The three dimensional structures of bovine angiogenin have been determined by ^{1}H 2D NMR spectroscopy.[14-17] Although the fold of the protein is very similar to those of pancreatic RNase A, angiogenin contains unique functional features that account for its characteristic enzymatic and angiogenic activities. [18] Bovine angiogenin (bANg) consists of 125 amino acids and has about 33% sequence identity with pancreatic RNase A. Also it has 64% amino acid sequence identity between bovine and human angiogenin. It has been reported that the pyrimidine-binding site of human angiogenin is blocked by glutamine 117, indicating that Ang must undergo a conformational change to bind and cleave RNA. The mechanism and nature of this change are still not known. In this study, we investigate the chemical shift variation of bovine angiogenin (bAng) upon binding with inhibitor phosphate using NMR spectroscopy to understand how nucleotide phosphate inhibitor can bind to bAng.

EXPERIMENTALS

Protein expression and purification

Bovine angiogenin was previously cloned[19] into a pET-21a(+) (Novagen) vector containing an IPTG inducible promoter and resistance to ampicillin. This vector was used to transform the E. coli strain Rosetta (DE3) pLysS for expression of bovine angiogenin.

Bovine angiogenin was expressed in an insoluble form. The insoluble pellet obtained after complete sonication of cells was dissolved in 0.1M Tris-HCl buffer (pH 8.0) containing 6M Gu-HCl, 1 mM EDTA, 0.1M NaCl and 10mM reduced dithiothreitol (DTT^{red}). The soluble extract was then slowly diluted for refolding with 0.1M Tris-HCl (pH 8.0), 1mM EDTA, 0.3mM oxidized glutathione(GSSG), 1.5mM reduced glutathione(GSH) and then incubated for 2 weeks at 4 ℃. The protein was purified using a cation-exchange FPLC column packed with SP-Sepharose Fast Flow (Pharmacia) and equilibrated with 25mM Tris-HCl (pH 8.0). Further purification was carried out by gel filtration column chromatography with HiLoad Superdex 75 pred grad column (Phamacia). The collected fractions from the gel filtration column were dialyzed against water for 24h at 4 ℃. Uniformly ^{15}N-isotopically labeled protein samples were prepared by growing cells in the M9 minimal media containing $^{15}NH_4Cl$ (Cambridge Isotope Laboratories) either with or without $^{13}C_6$-D-glucose (Cambridge Isotope Laboratories) as the sole source of nitrogen and carbon. The final buffer consists of 50 mM sodium phosphate and H_2O for bovine angiogenin (pH 5.5). Sodium azide (0.02%, mass.vol) was added to prevent microbial growth. All protein NMR experiments were performed on 1.0 mM ^{15}N-labeled and $^{13}C/^{15}N$-labeled bovine angiogenin samples.

NMR spectroscopy

All NMR experiments were recorded at 298K on a Bruker Avance 800 MHz spectrometer at Korea Basic Science Institute (KBSI). The backbone assignments were accomplished using the HNCACB and CBCA(CO)NH spectra. The sequential assignment by triple resonance data were confirmed by ^{15}N-edited TOCSY-HSQC and ^{15}N-edited NOESY-HSQC spectra as identifying $d_{NN(i, i+1)}$ and $d_{\alpha N(i, i+1)}$ NOEs. The assignment was started by picking cross-peaks in a two-dimensional ^1H-^{15}N HSQC, to obtain HN(i) and $^{15}N(i)$ resonance frequencies.

In order to investigate the chemical shift variation of bAng upon binding with inhibitor phosphate using NMR spectroscopy, HSQC spectra of bAng were acquired in phosphate buffer and in water, with the ^1H carrier set coincident with the water resonance and ^{15}N frequency set to 115 ppm; spectral widths were 8802.817 Hz and 4055.150 Hz in the t_2 and t_1 dimensions with 2048 and 256 complex points in each dimension respectively. A recycle

delay of 2.5 s was used in all NMR experiments. NMR data was processed with NMRPipe and visualized with Sparky.

RESULTS AND DISCUSSION

Originally, the three dimensional structure of bovine angiogenin as shown in Fig. 1 has been determined by proton 2D NMR techniques [20] without isotope labeling. In this study the backbone assignments were accomplished using the HNCACB and CBCA(CO)NH spectra. ^{1}H-^{15}N HSQC spectrum is shown in Fig. 2. HSQC spectrum was successfully completed because the spectra were well resolved and chemical shift dispersions were suitable for successful assignments.

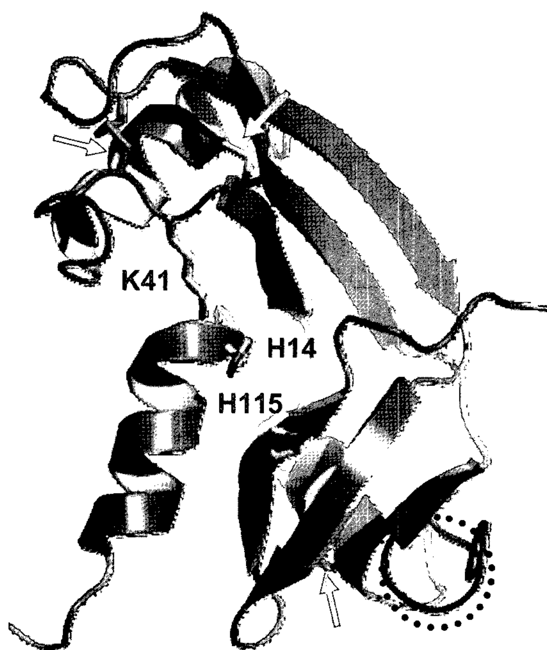

Fig. 1. The three dimensional structure of bAng. Ribonucleolytic active site consists of residues, His14, His115, and Lys4. Cell binding site is the interaction site with endothelial cell (circle) Angiogenin has three disulfide bridges indicated with arrows. This figure was prepared with PyMol.

In order to investigate the chemical shift variation upon binding with phosphate ions, HSQC spectra of bAng in phosphoate buffer and that in water were acquired. Fig. 3 shows the peaks which show the big difference in chemical shift upon binding with phosphate ions.

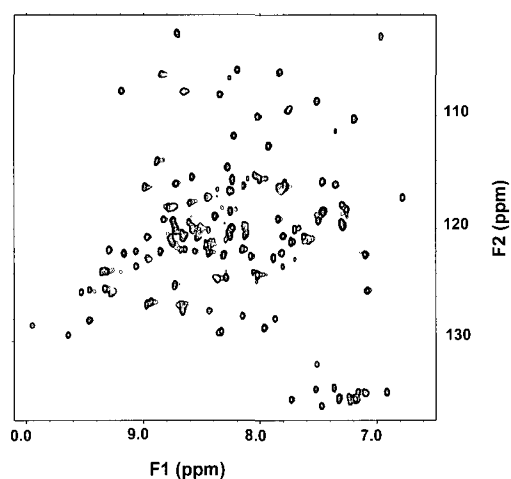

Fig. 2. The ^{1}H-^{15}N HSQC spectrum of bovine angiogenin in 50 mM sodium phosphate buffer.

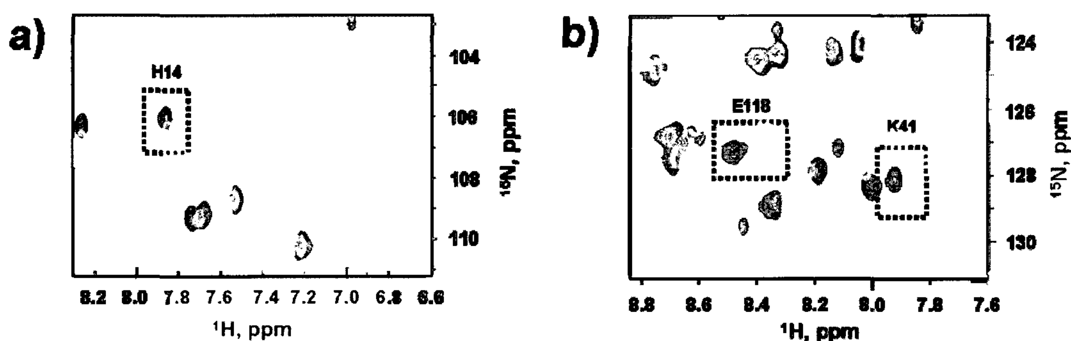

Fig. 3. HSQC spectra which shows the difference of chemical shift upon binding with phosphate ions. Peaks in black are the spectra of bAng in phosphate buffer and peaks in gray are the spectra of bAng in water.

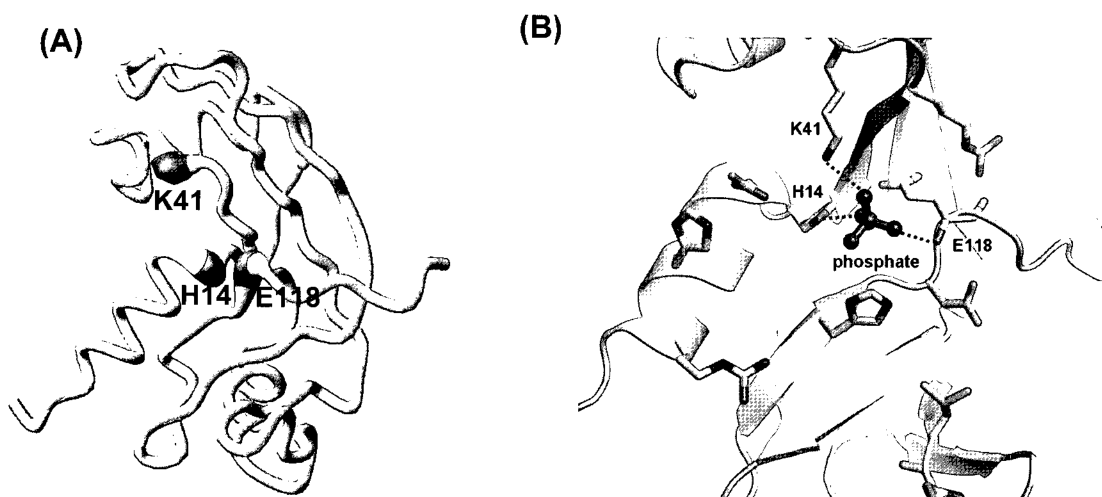

Fig. 4. (A)Spatial location of chemical shift variations. Residues with big chemical shift variations over 0.1 ppm are shown with larger ribbon diameter than the rest of the protein backbone. The figure was prepared with MolMol. (B) Docking model of bAng with phosphate ion.

Fig. 4A shows the spatial location of chemical shift variation upon binding with phosphate ions. His14, Lys41, and Glu118 near the ribonucleolytic site show big variations over 0.1 ppm. Chemical shift variation was calculated using the equation: $\Delta\delta av = \{0.5[\Delta\delta(^{1}H^{N})^{2} + (0.2\Delta\delta(^{15}N))^{2}]\}^{1/2}$ where $\Delta\delta(^{1}H^{N})$ and $\Delta\delta(^{15}N)$ are the chemical shift differences for the $^{1}H^{N}$ and ^{15}N atoms between two spectra [21]. These results imply that these residues might form a stable hydrogen bonding with phosphate ions. We proposed the docking model of bANg with phosphate ions as shown in Fig. 4B. Possible hydrogen bindings between the side chains of His14 and Lys41 in bAng and the phosphate groups are dictated. Also, backbone amide proton of Glu118 and the phosphate ion can make a stable hydrogen bonding. All these residues are the key residues involved the ribonucleolytic activity.

Clinical studies have revealed increase of Ang expression to be associated with

progression of several human cancers. Therefore, Ang can be a promising target for new anticancer drugs. In the present study, we have determined the binding sites of bAng with phosphate ion, which can be component of the nucleotide inhibitors. Phosphate occupies the catalytic site of bAng. In order to study the conformational and dynamical change upon binding with phosphate ions, spin relaxation experiments will be performed.

Acknowledgments

This work was supported by a Molecular and Cellular BioDiscovery Research Program grant (M10301030001-05N0103-00110) from the Ministry of Science and Technology. Sunhee Baek is supported, in part, by the second BK21 (MOE). We thank NMR Research Program of Korean Basic Science Institute (Y.H.J., granted by Korean Ministry of Sicence and Technology) for providing us 800 MHz NMR instrument time.

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