J. Biochem. Mol. Biol. Vol. 29, No. 4 pp. 353~358 July 31, 1996

# Interaction between a Blood Vessel-Inducing Protein Angiogenin and Its Binding Protein Actin

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(Received March 8, 1996)

**Abstract:** Bovine angiogenin (bAng) is a potent blood vessel inducing protein purified from cow milk. Fluorescence spectroscopy has been used to study the interaction of bAng with actin in 50 mM Tris-HCl, pH 7.5, and 1 mM $CaCl_2$ at 25℃. Actin contains four tryptophans but bAng contains no tryptophans. A 50% decrease in intrinsic fluorescence accompanied formation of the bAng/actin complex. By contrast, the interaction of RNase A, a homologous protein to bAng, with actin results in about 10% quenching of the fluorescence. Fluorescence titration experiments were performed by adding increasing concentrations of bAng (0~1.0 μM) to a constant concentration of actin (0.1 μM), and the dissociation constant $K_d$ for the bAng/actin complex and the stoichiometry n were measured as 20± 1 nM and 1.0± 0.1, respectively. These results suggest that the interaction between bAng with actin is specific and that quenching of actin fluorescence has occurred in the bAng/actin complex. The bAng binding sites of actin are discussed in the results of this study, and we propose that Trp-80 in the small domain of bovine actin is responsible for the bAng/actin binding.

**Key words:** actin, angiogenin, angiogenesis.

Angiogenesis, the process of development of new blood vessels, is essential in reproduction, development, and wound repair, and it is highly regulated under these conditions. However, many diseases such as arthritis, diabetic retinopathy, psoriasis, Kaposi's sarcoma, and in particular solid tumors are driven by persistent unregulated angiogenesis (Folkman and Shing, 1992). Inhibition of angiogenesis is a potentially valuable strategy to prevent and treat angiogenesis-dependent diseases (Folkman, 1995).

Angiogenin (Ang) is a potent blood vessel inducing protein originally purified from the conditioned media of cultured colon adenocarcinoma cells (HT-29) (Fett *et al.*, 1985). It was later detected and purified from normal human (Shapiro *et al.*, 1987), bovine (Bond and Vallee, 1988), rabbit (Bond *et al.*, 1993), pig (Bond *et al.*, 1993) and mouse sera (Bond *et al.*, 1993), and bovine milk (Maes *et al.*, 1988). Extensive studies of Ang on its enzymatic (Shapiro *et al.*, 1986; Lee and Vallee, 1989) and biological activities (King and Vallee, 1991; Rybak *et al.*, 1992; Tschesche *et al.*, 1994; Matousek *et al.*, 1995; Wu *et al.*, 1995), expression in tissues and cells (Rybak *et al.*, 1987; Li *et al.*, 1994; Moenner *et al.*, 1994), interaction with cells (Bicknell and Vallee, 1988, 1989; Badet *et al.*, 1989; Soncin, 1992; Hu *et al.*, 1991, 1994; Jimi *et al.*, 1995), structure (Acharya *et al.*, 1994, 1995; Reisdorf *et al.*, 1994), mechanism of action (Hallahan *et el.*, 1991, 1992; Moroianu and Riordan, 1994), and polyclonal/monoclonal antibodies (Blaster *et al.*, 1993; Olson *et al.*, 1995) have been carried out.

Ang binds to human placental ribonuclease inhibitor (PRI), and its enzymatic and biological activities are inhibited by PRI (Shapiro and Vallee, 1987). In addition, Ang binds to a 42-kDa dissociable cell surface actin of CPAE cells (Hu *et al.*, 1991), and its binding protein AngBP is a smooth muscle type of α-actin (Hu *et al.*, 1993). Binding of Ang to the cell surface actin results in the stimulation of cell-associated proteolytic activities which promote the degradation of basement membrane and extracellular matrix (Hu and Riordan, 1993; Hu *et al.*, 1994). It, therefore, has been suggested that the interaction between angiogenin and cell surface actin is an essential step in the angiogenesis process induced by Ang and in the process of endothelial cell invasion. Recently, Olson *et al.* (1995) reported that actin prevents tumor growth *in vivo* without any toxic side effects. Studies on the interaction of Ang with actin are important for an elucidation of the biological role of Ang and in particular the rational design of antiangiogenesis agents based on actin.

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In this study, we further characterized the interaction of bovine angiogenin (bAng) with actin by examining tryptophan fluorescence as a probe of changes in tryptophan environments in the bAng/actin complex.

## Materials and Methods

### Materials

Bovine milk was obtained from the dairy farm at Chungbuk National University. Trizma base and bovine muscle actin were from Sigma Chemical Co. (St. Louis, USA). Sep-Pak C18 cartridges were from Millipore Corp. (Bedford, USA). SP-Sepharose and Mono S were from Pharmacia Biotech. (Uppsala, Sweden). All other chemicals used were of analytical grade. All water and buffers were passed through Sep-Pak C18 cartridges to remove trace levels of RNases.

### Purification of bovine angiogenin from bovine milk

Bovine angiogenin was isolated from bovine milk by a three-step procedure (SP-Sepharose cation-exchange chromatography, Mono S FPLC, and C18 HPLC) essentially as described for its purification from bovine plasma (Bond and Vallee, 1988). Amino acid analyses and automated sequence analyses of the final purification were carried out at the Korea Basic Science Institute (Taejon, Korea), and its identity as bAng was confirmed.

### Quantitation of bovine angiogenin in each purification step

The polyclonal antibody against bAng was produced as described by Park (1995). In order to quantitate bAng in unfractionated samples and a pool of fractionated samples during the purification procedure, a sandwich enzyme-linked immunosorbent assay (ELISA) was carried out as described by Park (1995). The concentration of bAng was calculated based on the standard curve of bAng.

### Biological assays

Angiogenic activity was assessed by the chick chorioallantoic membrane (CAM) assay as described by Fett *et al.* (1985) except that the CAM was visualized after injecting with 10% fat emulsion.

### Mass spectrometry

Mass spectra of bAng were obtained with a Kratos Kompact MALDI2 at the Korea Basic Science Institute (Taejon, Korea).

### Absorption spectra

Absorption spectra were recorded with a Beckman DU70 Spectrophotometer and 0.1 μM bAng dissolved in 50 mM Tris-HCl, pH 7.5. The concentration of bAng was determined by amino acid analysis (PicoTag method; Waters Associates). The value of 8575 $M^{-1}cm^{-1}$ was determined as the molar extinction coefficient at 278 nm.

### Fluorescence measurements

Fluorescence data were recorded on a Hitachi Model F-3000 fluorescence spectrophotometer equipped with a thermostated cell holder maintained at 25℃. A 3 ml cuvette of 1 cm path length was used for recording fluorescence spectra. Excitation was at 285 nm, and emission was recorded from 300 to 450 nm with a 310-nm cut-off filter.

For fluorescence titration experiments, small aliquots of a stock bAng solution were added to 0.1 μM actin in 50 mM Tris-HCl, pH 7.5, and 1 mM $CaCl_2$ at 25℃. Concentrations of actin were determined spectrophotometrically using the molar extinction coefficient at 290 nm, $2.49\times10^4$. Excitation was at 285 nm, and emission was monitored at 345 nm with a 310-nm cut-off filter. The stoichiometries, n, and the equilibrium dissociate constants, $K_d$, were determined by fitting the data to

$$[L_t]\alpha = K_d/(1-\alpha)+n[Pt] \quad (1)$$

where $[L_t]$ is the concentration of added bAng, and $[P_t]$ is the concentration of actin. α is the fraction of actin sites occupied by bAng, and is given by $\alpha = F_{max}-F/(F_{max}-F_{min})$, where F is the fluorescence intensity measured, $F_{max}$ is the fluorescence intensity at the start of the titration, $F_{min}$ is the fluorescence intensity at the saturating concentration of bAng.

## Results and Discussion

### Purification, quantitation, and identification of bAng from bovine milk

bAng was isolated from bovine milk and quantitated by the sandwich enzyme-linked immunosorbent assay as shown in Table 1. The yield was 2.1 mg of the bAng per liter of bovine milk, and the final preparation contained 28% of the bAng immunoreactivity measured in the initial homogenate after defatting by centrifugation. Assuming that the immunoreactivity in the homogenate was primarily due to the bAng, the overall extent of purification was about 2000-fold. Previously, the placental ribonuclease inhibitor (PRI) binding assay (Bond, 1988) was used to quantitate bAng in chromatographic fractions only since this assay cannot definitively detect Ang in unfractionated samples which contain a significant amount of RNase(s). Immunological assays used in this study, however, quantitate bAng

**Table 1.** Purification of bovine angiogenin

| Step | Vol (ml) | Protein[a] (mg) | bAng[b] (mg) | bAng/protein (mg/mg) | Purification (fold) |
|---|---|---|---|---|---|
| Cow milk | 3000 | 43.800 | 22.4 | $5.1\times10^{-4}$ | 1 |
| SP-Sepharose | 45.5 | 36000 | 21.0 | $5.8\times10^{-4}$ | 1.1 |
| Mono S | 41.6 | 21.8 | 9.5 | 0.44 | $8.6\times10^{2}$ |
| C18 | 19.2 | 6.2 | 6.2 | 1 | $2.0\times10^{3}$ |

[a]Protein quantitations were obtained by amino acid analysis for the C18 sample and by the procedure of Bradford (1976) for the remainder.
[b]bAng quantitations were determined by the sandwich enzyme-linked immunosorbent assay (Park, 1995).

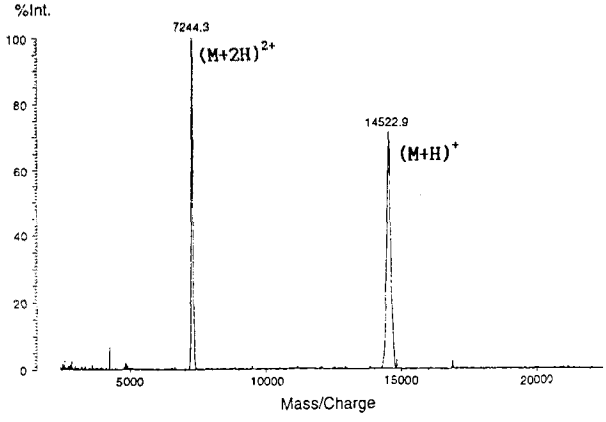


**Fig. 1.** The matrix assisted laser desorption ionization-time of flight mass (MALDI-TOF) spectrum of bAng.

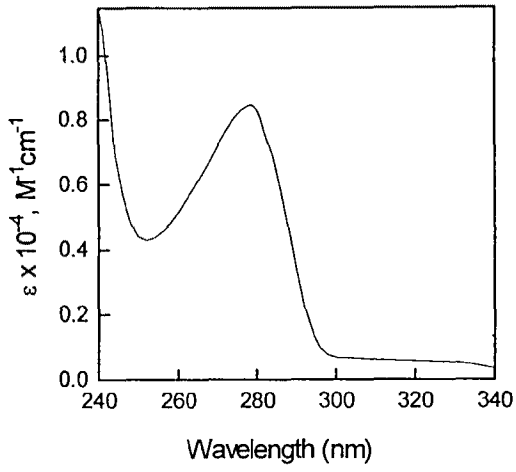


**Fig. 2.** UV absorption spectrum of bAng. bAng (0.1 μM) was in 50 mM Tris-HCl, pH 7.5. The concentration of bAng was determined by amino acid analysis.

in unfractionated samples as well as in chromatographic fractions as shown in Table 1. The final C18 HPLC purified bAng was at least 98% pure as judged by SDS-PAGE (Laemmli, 1970), revealing a single diffuse band of apparent molecular weight 16500 Da, which does not correspond to the calculated molecular weight (14595 Da) of bAng by the amino acid sequence (Bond and Strydom, 1989). The reason why bAng runs anomalously in SDS-PAGE is not known. Automated Edman degradation of the amino-terminal 7 residues indicates a sequence identical with that previously reported for the bovine serum bAng (Bond and Strydom, 1988): Ala-Gln-Asp-Asp-Tyr-Arg-Tyr. The purified bAng was tested for angiogenic activity in the chick embryo CAM assay. bAng (120 ng/egg) was active when compared to water controls (59% positive responses).

### Physical characterization of bAng

Aliquots of the final purification were analyzed by matrix assisted laser desorption-time of flight (MALDI-TOF) mass measurement. Fig. 1 shows the MALDI-TOF mass spectrum of bAng, and the peak corresponding to singly and doubly charged ions was observed. The MALDI-TOF mass spectrometric analysis reveals a molecular mass of 14522. The experimentally determined mass of bAng well agrees with a calculated molecular mass from the amino acid sequence, 14595 Da (Bond and Strydom, 1989). This result suggests that bAng is not post-translationally modified. In addition, the reason why bAng runs anomalously in SDS-PAGE is not due to its glycosylation but probably due to its basic property (pI>10.5; Bond and Vallee, 1988). It is worthy to note that the protein mobility in denaturing gel electrophoresis is not strictly a measure of the chemical mass since its mobility is influenced by both the amount of bound SDS and conformational features of the protein (Scoble *et al.*, 1993).

The UV absorption spectrum of bAng (Fig. 2) reveals a maximum at 278 nm with a molar absorptivity of 8575 $M^{-1}cm^{-1}$. This absortivity is slightly lower than a calculated value (9162 $M^{-1}cm^{-1}$) from knowledge of its amino acid composition (Gill and von Hippel, 1989).

### Interaction of bAng with actin

Knowledge of the nature of the Ang/actin interaction is of great importance to angiogenin function, since angiogenin-induced neovascularization on the CAM is

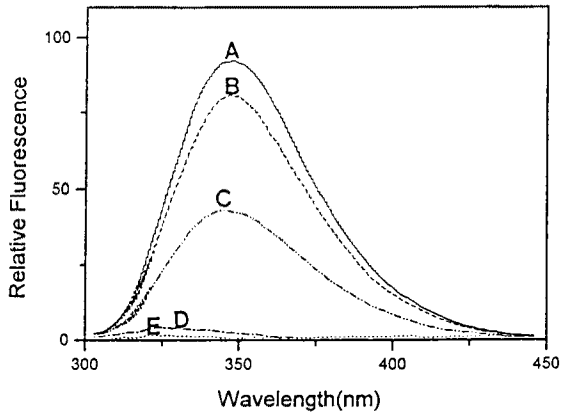


**Fig. 3.** Fluorescence emission spectra of actin (A), actin/RNase A (B), actin/bAng (C), bAng (D), and RNase A (E). All proteins were 0.1 μM in 50 mM Tris-HCl, pH 7.5, and 1 mM $CaCl_2$ at 25℃. Excitation was at 285 nm, and emission was monitored with a 310-nm cutoff filter.

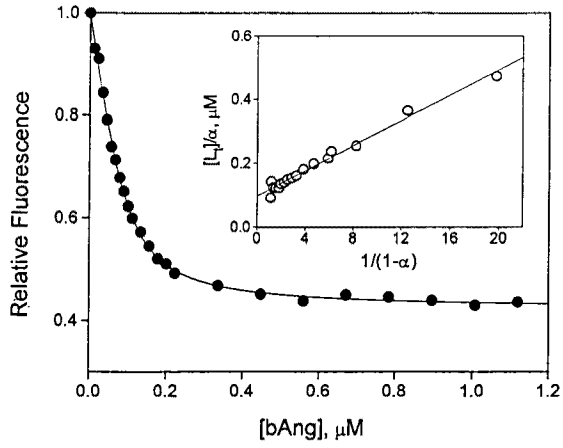


**Fig. 4.** Titration of actin (0.1 μM) with bAng using fluorescence as a signal of binding. The solid line of the inset plot has been calculated with the best-fit parameters to Eq. (1): $K_d = 20 \pm 1$ nM and $n = 1.0 \pm 0.1$. The curve has been calculated with these constants and the Eqs. (2) and (3).

inhibited by actin and the interaction between angiogenin and cell surface actin is an essential step in the angiogenin-induced angiogenesis. In addition, a basis for the design of the inhibitor to control angiogenin-induced angiogenesis can be provided from information on the Ang/actin interaction.

In this study, we have therefore examined the tryptophan fluorescence of actin as a means of monitoring the Ang/actin interaction. The fluorescence emission spectra of bAng, actin, and the bAng/actin complex are shown in Fig. 3. Actin contains four tryptophans and has a fluorescence spectrum with a maximum at 345 nm. The fluorescence of bAng is negligible at 345 nm since bAng contains no tryptophans. The fluorescence of the bAng/actin complex is quenched 50% relative to that of the sum of the two proteins. By contrast, that of the RNase A/actin complex is quenched slightly (~10%). RNase A is a homologous protein to bAng with 33% sequence identity (Bond and Strydom, 1989). These results suggest that the interaction between bAng and actin is specific.

Since there is a significant fluorescence change accompanying formation of the bAng/actin complex, fluorescence titration experiments were performed by adding increasing concentrations of bAng (0~1.0 μM) to a constant concentration of actin (0.1 μM). A plot of the fluorescence change *vs.* the concentration of the bAng is shown in Fig. 4. These data were used to construct the Webb plot (Bagshaw and Harris, 1987) in the inset of Fig. 4, which was analyzed according to Eq. (1) in Materials and Methods. A linear least-squares analysis gives $K_d = 20 \pm 1$ nM and $n = 1.0 \pm 0.1$. The curve in Fig. 4 has been calculated with these constants and the following relationship:

$$F = F_{max} - (F_{max} - F_{min})[L_b]/n[P_t] \quad (2)$$

where $[L_b]$ is the bound ligand concentration and $[P_t]$ is the total protein concentration. If the binding can be described by a single dissociation constant, $K_d$, then

$$2[L_b] = n[P_t] + [L_t] + K_d - \{(n[P_t] + [L_t] + K_d)^2 - 4n[P_t][L_t]\}^{1/2} \quad (3)$$

The $K_d$ value for the binding of bAng to actin is one order of magnitude higher than the value previously reported for the bAng/actin binding interaction, 0.5 nM (Hu *et al.*, 1993). The latter value was determined by a cross-linking technique and computed on the assumption that (i) the yield of crosslinking of bound $^{125}$I-Ang to actin is constant and occurs much faster than dissociation of the $^{125}$I-Ang-actin and (ii) the disappearance of free $^{125}$I-Ang and free actin also occurs much faster than dissociation of the $^{125}$I-Ang-actin. The validity of the assumptions listed is not certain but the differences could be due to different techniques employed to determine its value. Badet *et al.* (1989) reported that $^{125}$I-hAng binds to a monolayer of CPAE cells with an apparent $K_d$ of 5 nM.

bAng exhibits ribonucleolytic activity, albeit of markedly different magnitude from that of RNase A (Bond and Vallee, 1988; Bond *et al.*, 1993). The ribonucleolytic activity of bAng, however, is necessary but not sufficient for angiogenic activity (Hallahan *et al.*, 1991). bAng was found to bind human placental ribonuclease inhibitor (PRI) in a 1 : 1 molar ratio, and a binding constant for the interaction of bAng with human PRI is 3.4 fM (Bond *et al.*, 1993).

It has been suggested that the actin binding sites of Ang involves the site containing a putative cell sur-

face receptor binding site that is distinct from the catalytic site (His-14, Lys-41, and His-115, bAng numbering) (Hu *et al.*, 1991). The region of bAng that encompasses residues 58~70 and 108~111 has been implicated as part of a cell surface receptor binding site (Acharya *et al.*, 1995). The Ang binding sites of actin, however, are not known.

It is reported that DNase I binds mainly to the small domain of actin (Kabsch *et al.*, 1985), and that the $K_d$ value for the binding of actin and DNase I is 10 pM (Mannherz *et al.*, 1980). In addition, according to the high resolution structure of the actin-DNase I complex (Kabsch *et al.*, 1990), the main contact between actin and DNase I involves the loop formed by residues Arg-39 to Gly-46 of subdomain 2 in the small domain. Since actin contains four tryptophans whereas bAng has none, the quenching of fluorescence in Ang/actin (Fig. 3) suggests that quenching of actin tryptophan fluorescence has occurred in the complex. Preliminary results (unpublished data) in this laboratory revealed that bAng competes with DNase I for actin, and thus suggested that tryptophan(s) in the small domain of actin could be responsible for the bAng/actin binding. Four tryptophans (Trp-79, Trp-86, Trp-340, and Trp-356) are located in the small domain of the rabbit skeletal muscle actin and only Trp-79 is located in the surface of the crystal structure (Kabsch *et al.*, 1990). This, in turn, suggests that the environment of actin Trp-80 (bovine numbering) has changed markedly in the bAng/actin complex. This is consistent with the finding in this study, showing that tryptophan fluorescence is quenched by bAng. The three-dimensional structure of the bAng/actin complex, however, is necessary to understand fully the contact between actin and Ang.

In summary, we showed by fluorescence measurements and titration experiments that bAng binds to actin in a specific manner, and determined the dissociation constant of the bAng/actin complex, $\bar{K}_d$, and the stoichiometry, n. A detailed kinetics of the bAng/actin interaction is our next interest.

**Acknowledgement**

This work was supported by the NON DIRECTED RESEARCH FUND, Korea Research Foundation, 1994 and in part by the Basic Science Research Institute Program, Ministry of Education, the Republic of Korea, 1995 (Project No. BSRI-95-3434). We thank Dr. Young-Soo Kim (Chungbuk National University) for help in production of polyclonal rabbit antisera to bovine angiogenin.

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