

# Role of the surface loop on the structure and biological activity of angiogenin

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**Angiogenin is a member of the ribonuclease superfamily that induces the formation of new blood vessels. It has been suggested that the surface loop of angiogenin defined by residues 59-71 plays a special role in angiogenic function (1); however, the mechanism of action is not clearly defined. To elucidate the role of the surface loop on the structure, function and stability of angiogenin, three surface loop mutants were produced in which 14 amino acids in the surface loop of RNase A were substituted for the 13 amino acids in the corresponding loop of angiogenin. The structure, stability and biological functions of the mutants were then investigated using biophysical and biological approaches. Even though the substitutions did not influence the overall structure of angiogenin, they affected the stability and angiogenic function of angiogenin, indicating that the surface loop of angiogenin plays a significant role in maintaining the stability and angiogenic function of angiogenin. [BMB reports 2009; 42(12): 829-833]**

## INTRODUCTION

Angiogenin belongs to the ribonuclease (RNase) superfamily and shares 33% sequence identity with bovine pancreatic RNase A (2). The number and disposition of disulfide bonds are highly conserved among members of the RNase superfamily, which include angiogenin, RNase A and onconase, indicating that disulfides play a common role in the formation and maintenance of the structures in this superfamily.

Angiogenin was first identified based on the ability to possess angiogenic activity and has been isolated from a wide range of different sources, including human carcinoma cells (3), human plasma (4), rabbit, pig and mouse blood serum (5) and bovine serum and milk (6). Although angiogenin shows

weak ribonucleolytic activity that is four to six orders of magnitude lower than that of RNase, this activity is essential for the biological function of angiogenin (1, 7-9). Conversely, RNase is not angiogenic.

RNase A is a representative member of the RNase superfamily that has been used as a model system to investigate the pathways of protein folding and the role played by disulfide bonds in regeneration of the native proteins from the reduced form (10-16). RNase A has four disulfide bonds (Cys26-Cys84, Cys40-Cys95, Cys58-Cys110 and Cys65-Cys72), while angiogenin has three disulfide bonds (Cys27-Cys82, Cys40-Cys93, and Cys58-Cys108), and is structurally similar to bovine pancreatic RNase A, with some exceptions. The most notable of these exceptions is that the Cys65 and Cys72 residues in the "surface loop" region (see Fig. 1) (from Ser-59 to Tyr-73) of RNase A are replaced in angiogenin with other residues and two intervening residues are deleted (17, 18). Along with the catalytic site for cleaving RNA, this surface loop region of angiogenin has been suggested to play a special role in angiogenic function (1). However, the mechanism of action of this region has not yet been clearly defined.

In this study, we produced three surface loop mutants of bovine angiogenin (bAng) and investigated the role of the surface loop on the structure, function and stability of angiogenin.

## RESULTS AND DISCUSSION

To examine the role of the surface loop of angiogenin on its structure, function and stability, wild-type bAng and three surface loop mutants (ARN (Angiogenin with an RNase loop), ARN-[C65P, C72L] and ANG-[P65C, L70C]) were produced in *E. coli* and then purified as described previously (19). To construct ARN, 13-residues of bAng (residues 59-71) were substituted with 14 residues of the surface loop of RNase A (residues 59-72). The Cys65 and Cys72 residues of the surface loop of ARN were substituted with proline and leucine, respectively, for construction of ARN-[C65P, C72L]. In the case of ANG-[P65C, L70C], the Pro65 and Leu70 residues of the surface loop of bAng were substituted with cysteine residues. All mutants were > 95% pure according to SDS-PAGE and zy-

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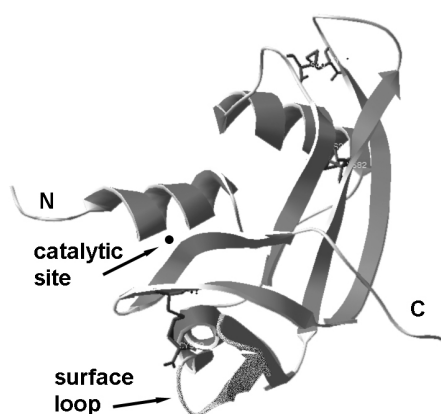
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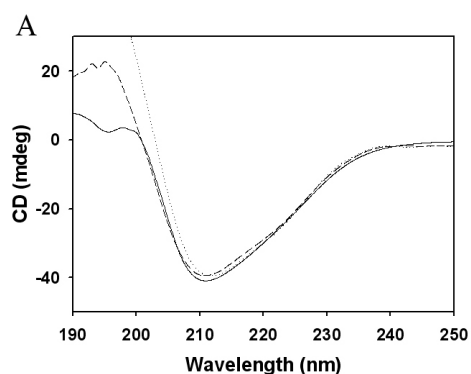
mogram electrophoresis, which is an extremely sensitive method of detecting ribonucleolytic activity (data not shown).

CD measurements in the far UV region (250-190 nm) yield data that provide information regarding the secondary structures of the protein molecule such as the amount of  $\alpha$ -helix,  $\beta$ -sheet,  $\beta$ -turn or random structure. As shown in Fig. 2A, the CD spectra of ARN, ARN-[C65P, C72L] and ANG-[P65C, L70C] were very similar to that of the bAng, indicating that substitutions on the surface loop do not affect the folding topology of the backbone structure. These spectra are characterized by a minimum near 212 nm.

To determine if modification of the surface loop influenced the stability of bAng, the melting temperatures ( $T_m$ ) of bAng and three mutants were measured using CD with a fixed wavelength at 220 nm. As shown in Fig. 2B, the three mutants are much less stable than bAng, as indicated by the  $T_m$  values being about 10°C lower than that of bAng. This indicates that the surface loop of angiogenin is well optimized, which likely has



**Fig. 1.** Ribbon diagram of bAng with the surface loop and catalytic site indicated. The three-dimensional structure of RNase A is similar to that of bAng, except for the surface loop.

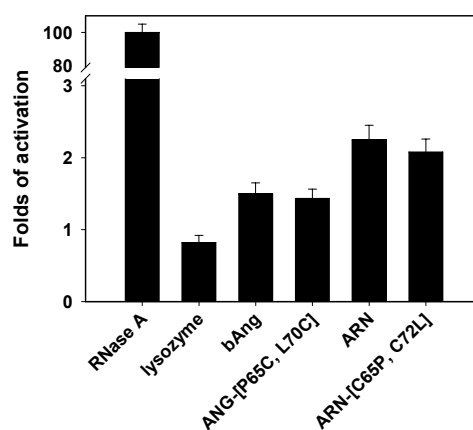


**Fig. 2.** (A) CD spectra of bAng and surface loop mutants recorded at 4°C in 20 mM Tris HCl, pH 8.0. (B) The thermal transition curves for bAng and surface loop mutants measured at 220 nm in 20 mM Tris HCl, pH 8.0 at a concentration of 0.5 mg/ml. (—) bAng; (···) ARN; (---) ARN-[C65P, C72L]; and (-·-) ANG-[P65C, L70C].

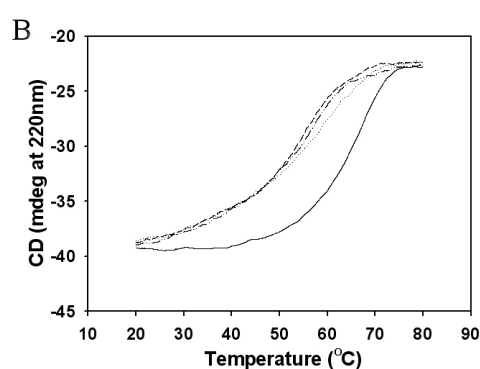
occurred through evolution. As a result, modification of the surface loop has a negative effect on the overall stability of angiogenin.

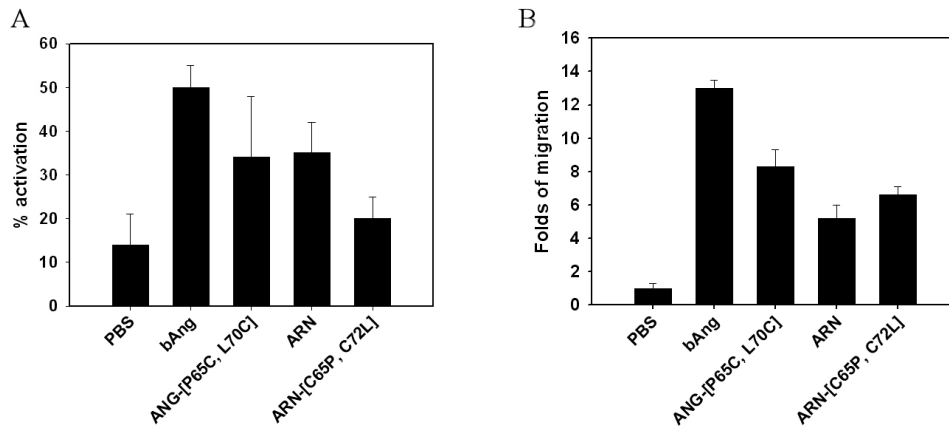
The ribonucleolytic activities of bAng and the surface loop mutants were also evaluated. Shapiro and Vallee reported that the ribonucleolytic activity of angiogenin is essential for its angiogenic activity (20). As shown in Fig. 3, the mutants containing the surface loop of RNase A (ARN, ARN-[C65P, C72L]) showed higher ribonucleolytic activities than those that had the surface loop of angiogenin (bAng and ANG-[P65C, L70C]). The existence of a disulfide bond did not affect the ribonucleolytic activity of the mutants, indicating that it is the sequence, not the constraint by a disulfide bond in the surface loop, that is important to the ribonucleolytic activity of angiogenin.

To determine if modification of the surface loop affects the angiogenic activity, a chick chorioallantoic membrane (CAM) assay (21) and a wound healing migration assay (22-24) were



**Fig. 3.** Ribonucleolytic activities of bAng and surface loop mutants. Ribonucleolytic activity was measured by a zymogram assay on a poly(C)-casting SDS-PAGE.





**Fig. 4.** Angiogenic activities of bAng and surface loop mutants as determined by (A) chorioallantoic membrane assay and (B) wound healing migration assay.

conducted. As shown in Fig. 4, all surface loop mutants constructed in this study showed lower angiogenic activities than bAng (Fig. 4). As in the case of ANG-[P65C, L70C], the addition of a disulfide bond in the surface loop of angiogenin had a negative effect on the angiogenic activity. However, this effect was less clear in the case of mutants with the surface loop of RNase A, as indicated by the opposite results obtained when CAM and wound healing migration assays of ARN and ARN-[C65P, C72L] were conducted.

Interestingly, the mutants with higher ribonucleolytic activity had lower angiogenic activity (Fig. 3, 4), which contrasts the previous finding that the angiogenic activity of angiogenin is dependent on its ribonucleolytic activity (25). This discrepancy could be due to the replacement of Asn61, which is an important residue for the angiogenic function of angiogenin.

Surface loops of angiogenin and RNase A differ in length and composition of amino acids. Specifically, RNase A has two cysteine residues for the [65-72] disulfide bond in the surface loop, but angiogenin does not contain cysteine residues in the same region. Raines *et al.* (26) examined the ribonucleolytic activity of a hybrid RNase A in which 15 residues in the surface loop were substituted with 13 analogous residues of angiogenin. The ribonucleolytic activity of this hybrid protein was 20-fold less than that of RNase A, but much greater than that of angiogenin. Interestingly, the substitution endowed RNase A with angiogenic activity.

In this study, we examined the effect of substitutions in the surface loop of angiogenin on the structure, function and stability of angiogenin. Even though the substitutions did not influence the overall structure of angiogenin, they did affect its stability and biological function (*i.e.* angiogenic activity). Therefore, that the results of this study indicate that the surface loop of angiogenin plays a significant role in maintaining the stability and angiogenic function of angiogenin.

## MATERIALS AND METHODS

### Construction of expression vectors

*E. coli* strain Rosetta (DE3)pLysS, which carries the pETbAng plasmid, was used to induce the expression of bAng (19). To construct the surface loop mutants, the DNA fragment encoding bAng was used as a template and the overlapping PCR was performed to produce substitutions in the surface loop. The final PCR products were digested with *NdeI* and *BamHI* and then ligated into the *NdeI*-*BamHI* restriction site of pET-21a (+) to construct the expression plasmids for the surface loop mutants, ARN, ARN-[C65P, C72L] and ANG-[P65C, L70C], respectively.

### Sample preparation

bAng and the surface loop mutants were expressed as inclusion bodies in *E. coli* Rosetta (DE3) pLysS cells (Novagen) and then purified using the procedures described by Jang *et al.* (19). The purity of the bAng and mutants was confirmed by reversed phase HPLC.

### Circular dichroism spectroscopy

Far-UV CD spectra were collected on a Jasco-810 spectropolarimeter (Jasco Co., Japan, Tokyo) at 4°C at a concentration of 0.5 mg/ml in 20 mM Tris-HCl (pH 8.0). Spectra were measured from 190 nm to 250 nm. The CD spectrum of the buffer was recorded and subtracted from the protein spectra.

### Thermal stability

The thermal stabilities of bAng, ARN, ARN-[C65P, C72L] and ANG-[P65C, L70C] were assessed by monitoring the change in CD with temperature at 220 nm. The temperature of a protein solution (0.5 mg/ml) in 20 mM Tris-HCl at pH 8.0 was increased in 1°C increments from 4 to 90°C.

### Zymogram electrophoresis

Zymogram electrophoresis (27) was used to measure the ribonucleolytic activities of bAng and the surface loop mutants. Briefly, samples were subjected to standard SDS-PAGE with the following modifications: the sample buffer did not contain a reducing agent, and the running gel was copolymerized with poly (C) (0.5 mg/ml), which is a substrate for bAng and RNase A. After electrophoresis, SDS was removed from the gel by washing (2 × 10 min) with 10 mM Tris-HCl buffer (pH 7.5) containing 2-propanol (20% v/v). Proteins were renatured by washing the gel for 10 min with 10 mM Tris-HCl buffer (pH 7.5), followed by 10 min with 0.10 M Tris-HCl buffer (pH 7.5) and then 10 min with 10 mM Tris-HCl buffer (pH 7.5). The gel was then stained with 10 mM Tris-HCl buffer (pH 7.5) containing toluidine blue (0.2% w/v) and destained with distilled and deionized water. Regions in the gel that contain ribonucleolytic activity appear as a clear band in a dark purple background.

### Chick chorioallantoic membrane (CAM) assay

To determine the angiogenic activity *in vivo*, a chick CAM assay was performed as previously described (21).

### Wound healing migration assay

The migration of human umbilical vein endothelial (HUVE) cells by bAng and surface loop mutants was determined using a scratchwound assay (22-24). HUVE cells were isolated from fresh umbilical cords and maintained in endothelial cell growth medium.

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