Crystal structure of bovine angiogenin at 1.5-Å resolution

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ABSTRACT The capacity of angiogenin (Ang) to induce blood vessel growth is critically dependent on its ribonucleolytic activity. Crystallography and mutagenesis of human Ang have previously shown that its pyrimidine binding site is obstructed by Gln-117, implying that a conformational change is a key part of the mechanism of Ang action. The 1.5-Å-resolution crystal structure of bovine Ang, in which glutamic acid is substituted for Gln-117, now confirms that a blocked active site is characteristic of these proteins. Indeed, the inactive conformation of bovine Ang is stabilized by a more extensive set of interactions than is that of human Ang. The three-dimensional structure of the putative receptor binding site is also well conserved in the two proteins. The Arg-Gly-Asp segment of this site in bovine Ang, which is replaced by Arg-Glu-Asn in human Ang, does not have a conformation typical of an integrin recognition site.

Angiogenin (Ang) is unique among angiogenic molecules in that it is a member of the pancreatic ribonuclease (RNase) superfamily (1) and, in fact, is a ribonucleolytic enzyme (2). Its enzymatic activity is extremely weak compared to that of the digestive RNases (2, 3) but is critical for its capacity to induce neovascularization (4, 5). Its in vivo substrate remains to be identified.

Ang was first isolated from human tumor cell conditioned medium (6) and subsequently from normal human plasma (7). Angs have also been purified from other mammalian sera (8, 9) and from cow’s milk (10). Most previous efforts to elucidate the structural basis for and relationship between enzymatic and angiogenic activities have focused on human Ang (hAng). Mutagenesis, proteolysis, and chemical modification studies have thus far identified several residues that play important or essential roles in catalysis or substrate binding and have at least partially accounted for the enzymatic differences between Ang and pancreatic RNase A (4, 5, 11–13). Moreover, they have demonstrated that angiogenic activity requires not only an intact catalytic site but also another region of Ang, thought to constitute a cell binding site.

Recently, hAng has been crystallized (14) and a 2.4-Å-resolution structure has been determined (15), which provides a basis for the unique functional properties of the protein. Most strikingly, it reveals that the site corresponding to the pyrimidine binding pocket of RNase A is blocked by Gln-117. Mutations of this residue to Ala and Gly increase enzymatic activity (16), implying that this conformation for Ang also exists in solution. The structure of native Ang in which access to the binding site is blocked presumably must undergo extensive reorientation in order to allow binding and cleavage of RNA. This conformational change might also serve to activate Ang at its target site in vivo.

In continuing efforts to further understand the structure–function relationships of Ang, we have now determined the x-ray structure of bovine angiogenin (bAng). This protein (Mr 14,595) has 64% sequence identity to hAng (10), virtually the same angiogenic potency, and, in general, similar enzymatic properties (see below). However, it has two differences in primary structure of potential significance: Glu-118 replaces Gln-117, and it contains a putative Arg-Gly-Asp (RGD) recognition element not present in hAng.

We here report that Glu-118 in bAng obstructs the pyrimidine binding site in precisely the same manner as does Gln-117 in hAng, and is, in fact, involved in a more extensive set of stabilizing interactions than its counterpart in hAng. Furthermore, the RGD sequence is not structurally similar to known integrin-dependent recognition sites. Importantly, the bAng structure has been determined to 1.5-Å resolution, which defines other interactions pertinent to the architecture of the active site in greater detail. It also provides a clearer view of additional functionally important regions, including the putative receptor binding site.

METHODS

bAng was isolated from unpasteurized milk essentially as described for its purification from plasma (8). Needle-shaped crystals (maximum dimensions, 0.7 mm × 0.3 mm × 0.3 mm) were grown at 16°C using the hanging drop method with a reservoir solution of 0.1 M sodium acetate/0.2 M ammonium acetate, pH 4.5/30% polyethylene glycol 4000. They belong to the orthorhombic space group P2₁2₁2₁; a = 30.69, b = 54.45, c = 74.75 Å with one molecule per asymmetric unit and 43% (vol/vol) solvent content. Diffraction data (AD-lab data set) were collected from native crystals (1.6 Å) using a Siemens area detector mounted on a Siemens rotating anode x-ray source using CuKα radiation (50 kV, 80 mA). Overall, 1900 frames of data were collected (0.25° per frame; crystal to detector distance, 10 cm; 2D angle, 35°; 120–130 sec per frame exposure). The data were processed using the XDS package (17). Higher-resolution data to 1.5 Å (SRS data set) were collected on station 9.5 of the Synchrotron Radiation Source (SRS; Daresbury, U.K.), using an 18-cm (diameter) MAR-research imaging plate system with an x-ray beam of 0.88-Å wavelength (0.2-mm collimator) from one crystal with 1.5° oscillations. The SRS data were processed using the MOSFLM package (A. Leslie, Medical Research Council, Cambridge, U.K.). The two data sets were scaled by using the program 3DSCALE (18) (Table 1). The structure was determined using the MERLOT molecular replacement programs (19) and the search was based on the hAng protein model (15) with bAng amino acid sequence changes incorporated into the model. The angles from rotation and translation function search gave a single, self-consistent set of translation vectors on the Harker sections. Cycles of positional and simulated annealing refinement using the X-PLOR package (20) and model building using FRODO (21) gave the refined model (using all data, 17,940

Abbreviations: Ang, angiogenin; bAng, bovine Ang; hAng, human Ang; SRS, Synchrotron Radiation Source.

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‡The atomic coordinates have been deposited in the Protein Data Bank, Chemistry Department, Brookhaven National Laboratory, Upton, NY 11973 (entry code 1AGI). This information is embargoed for 1 year (coordinates) from the date of publication.
Table 1. bAng x-ray data collection statistics

<table>
<thead>
<tr>
<th>Data set</th>
<th>N_m*</th>
<th>N_i</th>
<th>R_sym</th>
<th>% complete</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5-A native (SRS)</td>
<td>76,423</td>
<td>16,542</td>
<td>9.0</td>
<td>80</td>
</tr>
<tr>
<td>((\lambda = 0.88) A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6-A native (AD-lab)</td>
<td>73,455</td>
<td>16,060</td>
<td>4.6</td>
<td>94</td>
</tr>
<tr>
<td>((\lambda = 1.54) A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5-A native (all)</td>
<td>149,878</td>
<td>18,103</td>
<td>7.1</td>
<td>87</td>
</tr>
</tbody>
</table>

*Number of measurements.
†Number of independent reflections.
\(R_{sym} = \sum_{i} |I_i(h)| / \sum_{i} |\langle I_i(h) \rangle|\), where \(I_i(h)\) is the \(i\)th measurement of reflection \(h\) and \(\langle I_i(h) \rangle\) is the mean of the intensity.

RESULTS AND DISCUSSION

Overall Structure. In general, the electron density map is of high quality (Fig. 1) and the entire structure is well-defined except for the three N-terminal residues, the four C-terminal residues, the side chains of Asp-60 and Arg-61, and the terminal portions of the side chains of Lys-20, Lys-83, Arg-90, and Arg-102, all of which have high temperature factors and are disordered. The structure of bAng has the "RNase A fold" (Fig. 2) and is very similar to that of hAng (15).

Fig. 3 shows the sequence alignment of bAng and hAng and indicates which bAng residues are either solvent-inaccessible or involved in crystal contacts. Compared to the human protein, bAng has single-residue extensions at both termini and no insertions or deletions. The rms difference between the \(C^a\) positions of 121 equivalent residues in the two crystal structures (bAng 2–122) is 1.24 Å. The regions that deviate most significantly are the N-terminal residues 2 and 3 (5.16 and 2.47 Å, respectively) and the C-terminal residues 120–122 (1.65, 5.07, and 9.54 Å, respectively). The difference in the C-terminal region is particularly noteworthy as discussed below. In hAng, residues 117–121 form a 3_\(\mu\) helix that is absent in bAng; instead, the corresponding segment appears to be flexible and disordered after the second residue, Ser-119. The amino acid sequence in this segment is also poorly conserved between the two Angs. Exclusion of these residues and amino acids 2 and 3 from the structural comparison reduces the rms deviation in \(C^a\) positions for the two proteins to 0.51 Å, indicating a high degree of similarity between the structures. Only five residues (21, 50, 68, 88, and 89) differ in position by >1.0 Å. All but one of these (Asn-50) are on a loop. Two peptide bonds in the Ang crystal structure adopt a cis conformation: those connecting Arg-38 with Pro-39 and Arg-90 with Pro-91.

Fig. 2. Polypeptide fold of bAng, drawn with the program Molscript (22). The secondary-structure elements shown [based on program DSSP (23)] contain the following residues: 4–15 (H1), 23–34 (H2), 42–48 (B1), 50–59 (H3), 63–66 (B2), 70–74 (B3), 77–85 (B4), 94–102 (B5), 104–109 (B6), and 112–117 (B7).

Fig. 3. Sequence alignment of hAng and bAng. Identities are indicated by uppercase letters. Hyphens indicate that there is no corresponding equivalent residue. The solvent-inaccessible residues [-<20 Å² of exposed surface, based on the program DSSP (23)] are shown by stars. Residues involved in direct lattice contacts in the crystals are indicated by % signs, whereas residues involved only in indirect lattice contacts are shown by plus signs.

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**DISCUSSION**

Fig. 1. Portion of a \(2|F_o| − |F_c|\) electron density map of bAng contoured at 1.0σ using the refined structure at 1.5-A resolution.
Pro-91 with Pro-92. Both bonds are also cis in the hAng structure (1ANG-PDB).

Pyrimidine Binding Site B1. The major, unexpected feature of the hAng crystal structure was the finding that Gln-177 blocks the site that is spatially analogous to the B1 pyrimidine binding pocket of RNase A. The same obstruction is seen for the corresponding residue, Glu-118, in bAng (Fig. 4). This is more dramatically evident when the inhibitor uridine vanadate is superimposed onto the B1 site of bAng by modeling (Fig. 5 Left): the Glu side chain is seen to pass through the uracil ring. The substitution of Gln by Glu in bAng is conservative: both of the side-chain H bonds (to N and OG1 of Thr-45; Table 2) of the human protein are maintained. In addition, Glu-118 is held in place by H bonds from its main-chain C=O and side-chain OE1 to Arg-43 (NH1 and NE, respectively). In hAng Arg-43 is replaced by Ile-42, which is not close to Gln-117. The Glu-118 carboxyl group also engages in two further interactions that are not present in the hAng structure: a water-mediated H bond to NZ of Lys-41 and a van der Waals contact with His-14 (3.13 Å from CE1). All of these interactions should help stabilize the obstructive conformation in bAng relative to that in hAng and may account for its severalfold lower enzymatic activity toward tRNA (M. D. Bond and R. S., unpublished results). Importantly, the bAng crystals used for structure determination were grown in acetate rather than the citrate/tartrate used for hAng (14) and they belong to a different space group, have lower solvent content, and utilize different residues as lattice contacts. Together with the results of mutagenesis experiments on Gln-117 of hAng (16), this essentially excludes the possibility that the observed blockage of the B1 site is a crystal artifact.

Although the native structures of hAng and bAng do not contain accessible B1 sites, both proteins cleave RNA and are highly specific for the base that would bind at this site (ref. 3; M. D. Bond and R. S., unpublished results). This implies that Ang undergoes a conformational change to open up the site.

Table 2. Selected H bonds in the enzymatic active site of bAng

<table>
<thead>
<tr>
<th>Residue</th>
<th>Group</th>
<th>Partner</th>
<th>Distance, Å</th>
</tr>
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<tr>
<td>His-14</td>
<td>N</td>
<td>O Phe-10</td>
<td>2.83</td>
</tr>
<tr>
<td>O</td>
<td>N Ile-47</td>
<td>2.74</td>
<td></td>
</tr>
<tr>
<td>ND1</td>
<td>O Thr-45</td>
<td>2.75</td>
<td></td>
</tr>
<tr>
<td>Lys-41</td>
<td>O Leu-36</td>
<td>3.10</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>OH Tyr-95</td>
<td>2.76</td>
<td></td>
</tr>
<tr>
<td>NZ</td>
<td>OD1 Asn-44</td>
<td>3.10</td>
<td></td>
</tr>
<tr>
<td>Thr-45</td>
<td>O OE2 Glu-118</td>
<td>2.90</td>
<td></td>
</tr>
<tr>
<td>O</td>
<td>See His-14</td>
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<tr>
<td>OG1</td>
<td>O E1 Glu-118</td>
<td>2.74</td>
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</tr>
<tr>
<td>His-115</td>
<td>N</td>
<td>O Gly-107</td>
<td>3.21</td>
</tr>
<tr>
<td>O</td>
<td>N Gly-107</td>
<td>2.84</td>
<td></td>
</tr>
<tr>
<td>Asp-117</td>
<td>N</td>
<td>O Val-105</td>
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<td>OG Ser-119</td>
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<tr>
<td>Glu-118</td>
<td>O</td>
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<td>2.91</td>
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<td>O</td>
<td>OE1 NE Arg-43</td>
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<tr>
<td>OE1, OE2</td>
<td>See Thr-45</td>
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<tr>
<td>Ser-119</td>
<td>N, O, OG</td>
<td>See Asp-117</td>
<td></td>
</tr>
</tbody>
</table>

*H bonds were calculated using a cutoff donor/acceptor distance of 3.3 Å and the angle criteria described in ref. 25.

**Distance between donor and acceptor atoms.**

![Fig. 4. Active site of bAng native refined structure at 1.5 Å, with H bonds indicated by dashed lines and water molecules indicated by the letter "W."](image1)

![Fig. 5. (Left) Crystal structure of active site of bAng with uridine vanadate (UV, in black) added by modeling as described (15). (Center) Corresponding model of hAng-UV complex. (Right) Crystal structure of RNase A-UV complex (24). Selected side chains are shown.](image2)
during its normal catalytic pathway, a view that is further supported by the results of mutagenesis and modeling studies on hAng (16). Information from both Ang x-ray structures is pertinent to this issue. In RNase A, Thr-45, Ser-123, and Phe-120 in RNase A have been proposed to interact with the pyrimidine moiety in the B₁ site, with the first two forming H bonds and the last making van der Waals or stacking contacts (see refs. 26 and 27). In both Angs, the corresponding Thr is positioned almost identically to Thr-45 in RNase and base specificity is similar to that of RNase. This would tend to rule out any substantial movement of this residue during the proposed conformational transition.

In contrast, Ser-119 of bAng and Ser-118 of hAng are positioned quite differently from Ser-123 of RNase A (Fig. 5). Hence, neither one would be expected to be part of the B₁ site. Consistent with this, mutations of Ser-118 in hAng do not alter specificity (5). At the same time, these mutations decrease overall activity to some extent, suggesting a catalytic role, perhaps indirect, for Ser-118 once the B₁ site has opened. This interpretation is not straightforward, however, since Ser-118(119) appears to help maintain the closed conformation by means of H bonds with Asp-116 (hAng)/Asp-117 (bAng) (ref. 15 and Table 2).

The replacement of Phe-120 in RNase A by Leu-115 in hAng has been thought to be another factor contributing to the lower enzymatic activity of Ang (28). bAng, like RNase A, contains a phenylalanine in this position. It is a residue that is located similarly in the three-dimensional structure. However, its phenyl ring is rotated by nearly 90° with respect to that of Phe-120 in RNase (Fig. 5); thus, if it interacts productively with the pyrimidine when the B₁ site opens, some reorientation would be required.

**Catalytic Center (P)**. The positions of the catalytic residues His-14, Lys-41, and His-115 in bAng are virtually identical to those of their counterparts in hAng and RNase A. Several interactions of these residues, not seen in the 2.4-Å-resolution hAng structure, can now be identified in the higher-resolution bAng structure (Fig. 4; Table 2). NZ of Lys-41 H bonds to OD₁ of Asn-44 (as in RNase A) and interacts via water molecules with NE₂ of Glu-13, NE₂ of His-14, and OE₂ of Glu-118. Numerous additional water molecules are found in this region. A water-mediated H bond that appeared to exist between His-114 and Asp-116 in hAng is clearly absent in bAng.

**B₂ Site**. A B₂ site for binding the base whose ribose group is engaged in the catalytic reaction has not yet been defined in hAng. Recent results of mutagenesis (29) demonstrate that Asn-71 and Glu-111 are components of this site in RNase A. The only potential H-bonding residues in the corresponding region of hAng are Asn-68 and Glu-108; mutagenesis indicates that Glu-108 plays only a minor role in activity (5), whereas the function of Asn-68 has not been examined as yet. This region differs significantly in bAng. Glu-109 is shifted somewhat from the position of Glu-108 in hAng and forms a H bond to NH₂ of Arg-6 (2.83 Å) that is absent in the human protein. Asp-69 in bAng replaces Asn-68 of hAng and deviates in orientation after the β-carbon (Fig. 6). In this regard it should be noted that the base specificity of bAng and hAng at this position differs markedly: bAng prefers G over A by several fold (M. D. Bond and R. S., unpublished results), whereas the inverse is true for hAng (3).

**Receptor-Binding Site**. Ang interacts with cultured vascular endothelial cells and elicits a number of responses that may be relevant to binding angiogenesis (30–32). These effects are presumably mediated through binding of Ang to cellular receptors. Mutagenesis, deamidation, and proteolysis studies on hAng indicate that the receptor binding site is distinct from the catalytic center and includes the segments 58–70 (loops 4 and 5 and β-strands B2 and B3) and 108–111 (loop 9) (3, 11–13).

[Interestingly, this region may also contain part of the B₂ subsite for RNA binding in Ang, as does the corresponding region of RNase A (see above).] Asn-61, Arg-66, and Asn-109 are the specific residues implicated thus far; replacements of each abolish angiogenic activity but have no significant effect on enzymatic activity.

All three of these residues are conserved in the bAng sequence, as Asn-62, Arg-67, and Asn-110. The conformations of the segments containing these residues are generally very similar [the rms deviation for Cα positions for residues 59–71 (Fig. 6) is 0.73 Å with the major difference being Gly-68 of bAng vs. Glu-67 of hAng (2.63 Å)]. Asn-62 and -110 in bAng are almost superimposable with the corresponding asparagines in hAng. The orientations of the Arg-67/66 side chains, however, differ markedly. This may reflect the inherent flexibility of these residues or the participation of the Arg-67 guanidino group of bAng in crystal lattice contacts.

This segment of bAng includes an RGD sequence (residues 67–69), a motif known to be a recognition element in many integrin-dependent cell adhesion processes (33). This motif is replaced by Arg-Glu-Asn in hAng and on this basis it has been proposed that the cellular interactions of the two proteins may differ (34). Thus far, however, no experimental evidence supports such a difference. Indeed, the angiogenic activities of bAng and hAng are equivalent (8) and, although bovine endothelial cells adhere to bAng through integrins, this is also true for their adhesion to hAng (32).

To examine this question in structural terms, we have compared the conformation of the RGD residues of bAng with those in two other proteins known to bind integrins, foot and mouth disease virus VP1 protein and γII- crystallin. The VP1 and γII-crystallin RGD structures are virtually identical (rms deviation for Cα atoms, only 0.09 Å) and differ markedly from RGD structures observed in proteins considered to lack integrin binding activity (e.g., xylene isomerase, thermolysin, tryptophan synthase, α-lytic protease; rms deviations, 0.53–0.89 Å) (see ref. 35). Although the RGD sequence in bAng is located on an exposed loop, as are its VP1 and γII-crystallin counterparts, its structure is quite distinct (Fig. 7) (rms deviations, 0.74 Å and 0.83 Å, respectively). These findings suggest that the RGD segment of native bAng is not aligned appropriately to participate in the common “RGD”-type interactions, although it remains possible that the conformation of this tripeptide changes as Ang binds to the cell surface.

**Other Regions of Interest**. Loop 7 of hAng (residues 85–92) forms part of the binding epitope for a monoclonal antibody
that is effective in preventing the establishment of human tumors in athymic mice (36, 37). It is also thought to be part of the contact region between Ang and placental RNase inhibitor, a potent inhibitor ($K_i = 0.7 \text{ fM}$) of the enzymatic and angiogenic activities of Ang (see ref. 38). Despite the relatively high degree of sequence similarity in this region of bAng and hAng, the loop conformations deviate considerably, perhaps due to the Ser/Pro substitution at position 89/88. This difference may account for at least part of the $>10^4$-fold preference of the antibody for hAng over bAng (36) and 20-fold faster dissociation of bAng from its complex with the RNase inhibitor (9).

As noted above, the structures of the C-terminal regions of bAng and hAng diverge dramatically beginning with bAng residue 120. Since the $3_1$ helix formed by this segment in hAng appeared to be a factor in creating or maintaining the obstructed conformation of the active site (15), such a difference was unanticipated. The positions of two amino acid side chains in bAng are incompatible with a helical conformation for this segment. Arg-43 lies close to the space occupied by the main-chain atoms of Phe-120 in hAng, and, hence, to accommodate the corresponding residue, Ile-121, the bAng polypeptide chain must adopt an alternative orientation. In addition, Arg-102 is shifted by several angstroms from the position of Arg-101 in hAng and is only 0.7 Å from that of the Phe-120 phenyl group in the human protein. On the basis of these findings, it would seem that the critical location of the obstructive Glu/Gln must be fixed primarily by interactions of this residue itself and of those immediately adjacent to it.\footnote{During preparation of this manuscript, a report was published providing assignments of secondary structure elements for bAng in solution as determined by NMR spectroscopy (34). The general fold observed is in agreement with that found in the crystalline state. Importantly, this similarity includes the C-terminal region, which is also disordered in the NMR structure.}

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