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Crystallization and preliminary X-ray diffraction studies of human angiostatin

Marta C. Abad and James Geiger*

Department of Chemistry, Michigan State University, East Lansing, MI 48824, USA

Correspondence e-mail: geiger@cem.msu.edu

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Angiostatin is an inhibitor of angiogenesis, the process by which new blood vessels are formed from pre-existing ones. The fact that tumor growth and metastasis dissemination are angiogenesis-dependent processes has awakened interest in angiogenesis inhibitors as anticancer treatment drugs. Angiostatin, a potent angiogenesis inhibitor, is currently in phase I clinical trials. Human angiostatin containing the first three kringle domains of plasminogen (K1-3) has been crystallized and high-resolution data were collected. The crystals belong to the $P4_12_12$ space group, with unit-cell parameters $a = b = 56.94$, $c = 192.97$ Å. A data set to a resolution of 1.75 Å with an overall $R_{\rm merge}$ and $I/\sigma(I)$ of 7% and 19.5, respectively, was obtained.

1. Introduction

Angiogenesis (Carmeliet & Jain, 2000), the sprouting of new blood vessels from pre-existing capillary beds, is a multi-step process consisting of endothelial cell proliferation and migration, basement membrane degradation and new lumen formation (Folkman & Shing, 1992). Pathological angiogenesis occurs in diabetic retinopathy and prolonged inflammation, as well as in tumor growth and metastasis (Folkman, 1995). In tumor growth, angiogenesis is critical since avascular tumors rarely grow beyond 2–3 mm^3; rapid growth is seen only after tumor vascularization (Hanahan & Folkman, 1996). Angiogenesis inhibitors have gained much public attention recently as potential anticancer agents and several such inhibitors are currently in clinical trials, including angiostatin (Phase I, Jefferson Hospital, Philadelphia, PA, USA). Angiostatin was originally isolated as an N-terminal fragment of plaminogen containing the first four kringle domains (Cao *et al.*, 1996). It was later found, however, that angiostatin containing only kringles one to three (K1-3) is twice as potent, with an ED_{50} (half maximal concentration) of 70 n*M* *versus* 135 n*M* for K1-4 (Cao *et al.*, 1996). Angiostatin K1-3 was found to be an extremely potent antitumor agent in animal models, decreasing brain tumor volume by 71% in mice, showing that angiostatin is not only an effective antitumor agent but that it can also cross the blood–brain barrier (Joe *et al.*, 1999).

The crystallographic structures of four of the five individual kringle domains in plasminogen have been determined previously (Chang *et al.*, 1998; Mathews *et al.*, 1996; Mulichak *et al.*, 1991; Rios-Steiner *et al.*, 2001; Wu *et al.*, 1991). Their binding modes for lysine-like ligands have been extensively studied both structurally (Chang *et al.*, 1998; Mathews *et al.*, 1996; Wu *et al.*, 1991) and by site-directed mutagenesis (McCance *et al.*, 1994). However, structures of multi-kringle domains have been elusive. This paper reports the crystallization and X-ray crystallographic data of human angiostatin (K1-3; residues 79–338, 29.5 kDa) to a resolution of 1.75 Å.

2. Materials and methods

The human angiostatin mutant N289E (this mutant prevents N-linked glycosylation) was expressed in *Pichia pastoris* and purified as described previously (Shepard *et al.*, 2000). The purified protein was buffer-exchanged into saline buffer and concentrated to 15 mg ml^{-1}.

3. Results and discussion

3.1. Crystallization

The protein was screened for crystallization using the hanging-drop vapor-diffusion method. The search for well diffracting crystals was performed using several sparse-matrix crystallization screens at two different temperatures, 298 and 277 K (Cudney *et al.*, 1994; Jancarik & Kim, 1991). A 4 µl hanging drop was equilibrated against 650 µl crystallization solution in a 1:3 protein to crystallization solution ratio. The best crystals were obtained at 277 K in a solution containing 10% polyethylene glycol (PEG) 20 000, 2% dioxane and 100 m*M* *N*,*N*-bis(2-hydroxyethyl)glycine (bicine) buffer pH 9.0. The crystals first appear

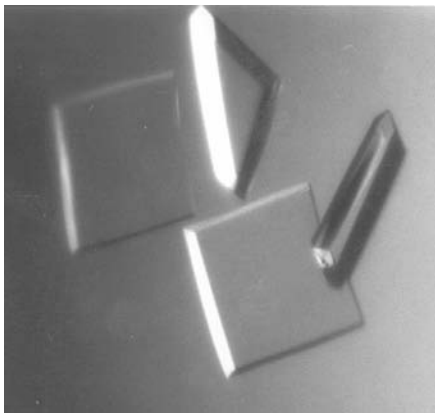

Figure 1
Tetragonal crystals of human angiostatin (K1-3). The crystals have dimensions of 0.4 × 0.4 × 0.2 mm.

overnight and reach maximum size in 3 days (Fig. 1).

3.2. Diffraction data collection

The crystal was transferred to a cryoprotectant solution containing 35%(*v*/*v*) glycerol, 10%(*m*/*v*) PEG 20 000, 2%(*v*/*v*) dioxane and 100 m*M* bicine pH 9.0 and quick-frozen in liquid nitrogen. X-ray diffraction data to a resolution of 1.75 Å were collected at the Structural Biology Center 19-ID beamline at the Advanced Photon Source (Argonne National Laboratory, Argonne, IL). One crystal was used for data collection; the data were collected using a custom-built 3 × 3 array (3072 × 3072 pixels) CCD area detector. The crystal-to-detector distance was 150 mm and 120° of data were collected (0.3° oscillation). Diffraction data were indexed and integrated using *HKL*2000 and *SCALEPACK* (Otwinowski & Minor, 1997).

The crystals are tetragonal and belong to the $P4_12_12$ space group, with unit-cell parameters $a = b = 56.94$, $c = 192.97$ Å. Assuming one molecule of angiostatin (29.5 kDa) per asymmetric unit the crystal volume per protein mass is 2.65, which corresponds to approximately 51% solvent content in the crystal. This value is within the range observed for protein crystals (Matthews, 1968). X-ray diffraction data was 99.6% complete, with an $R_{\rm merge}$ of 0.070 for 30 370 unique reflections from a total of 217 983 measured reflections. Detailed data-collection statistics can be found in Table 1. Structure determination is in progress using molecular-replacement methods.

Table 1
Data-collection statistics.

Data were collected at the Advanced Photon Source, Structural Biology Center ID19 beamline. Values in parentheses refer to the highest resolution shell.

Wavelength (Å)	1.0332
Resolution range (Å)	50–1.75 (1.81–1.75)
Completeness (%)	92.8 (83.0)
$I/\sigma(I)$	19.5 (5.1)
$R_{\rm merge}$† (%)	7.0 (34.1)
Unique reflections	30370
Measured reflections	217983

† $R_{\rm merge} = \sum_I |I_I - \langle I\rangle|/\sum\langle I\rangle$, where I_I is an individual intensity measurement and $\langle I\rangle$ is the average intensity for this reflection, with summation over all data.

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