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Crystallization and preliminary X-ray crystallographic studies of mouse autocrine motility factor

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Mouse autocrine motility factor (mAMF), a tumour-secreted cytokine that stimulates cell migration *in vitro* and metastasis *in vivo*, has been crystallized by the hanging-drop vapour-diffusion method. The crystals belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 69.97, b = 115.88, c = 73.27 Å, β = 101.76°. There are two subunits (one dimer) per asymmetric unit. Complexes with four-, five- and six-carbon carbohydrate phosphate inhibitors have also been crystallized. The crystals diffract to at least 1.8 Å resolution and are suitable for X-ray structure analyses at high resolution.

1. Introduction

Autocrine motility factor (AMF) was originally identified by its ability to stimulate directional motility (chemotaxis) and random motility (chemokinetics) of AMF-producing tumour cells (Liotta *et al.*, 1986). AMF stimulates cell motility *via* a receptor-mediated signalling pathway involving morphological changes, receptor phosphorylation, a pertussis-toxin-sensitive G-protein activation, inositol phosphate production, protein kinase C activation and enhanced production of a metabolite of arachidonic acid (Silletti & Raz, 1996). Recently, another role of AMF has been revealed: it stimulates AMF-producing tumour-cell motility in an autocrine manner and acts as a paracrine factor to vein endothelial cells to induce angiogenesis with cell-motility stimulation. It may facilitate metastasis through these effects during the metastasis phase (Funasaka *et al.*, 2001).

Partial amino-acid sequencing of mouse AMF (Watanabe *et al.*, 1996) and full-length cDNA cloning of human AMF (Niinaka *et al.*, 1998) have identified that the AMF is identical to the extracellular cytokines neuroleukin (NLK; Gurney *et al.*, 1986; Chaput *et al.*, 1988) and maturation factor (MF; Xu *et al.*, 1996) and, interestingly, to the intracellular enzyme phosphoglucose isomerase (PGI; Gracy & Tilley, 1975). PGI [also known as glucose 6-phosphate isomerase (GPI) and phosphohexose isomerase (PHI)] is a key enzyme in glycolysis and gluconeogenesis that catalyses the second step of glycolysis, the interconversion of glucose 6-phosphate (G6P) and fructose 6-phosphate (F6P). In recent years, several other protein factors have been identified as being identical to AMF: an antigen in rheumatoid arthritis (Matsumoto *et al.*, 1999) and sperm agglutination (Yakirevich & Naot, 2000) and a novel serine-protease inhibitor (MBSPI; Cao *et al.*, 2000). Thus, PGI not only acts as a housekeeping enzyme of sugar metabolism inside the cell, but also exhibits various properties of cytokines (AMF, NLK, MF and several other protein factors) outside the cell. Site-directed mutagenesis studies at the sugar-binding sites of human AMF/PGI have resulted in an abridging of the cytokine activity of mutant AMFs, suggesting that the regions important for enzymatic function overlap those for cytokine function (Tanaka *et al.*, 2002).

Several crystal structures of mammalian (human, rabbit and pig) AMF/PGIs complexed with sulfate ion occupying the binding site of the substrate phosphate (Read *et al.*, 2001; Davies & Muirhead, 2002) and with carbohydrate phosphate inhibitors are available (Jeffery *et al.*, 2000; Tanaka *et al.*, 2002), but a high-resolution structure of AMF/PGI complexed with a carbohydrate phosphate inhibitor is not. In the absence of the high-resolution structure of AMF/PGI complexed with an inhibitor, the structural image of the AMF–inhibitor interactions in the presence of water molecules, which is essential for lead compound design of more effective AMF inhibitors, is not sufficiently detailed. Here, we report the crystallization of mouse AMF (mAMF) in the absence and presence of carbohydrate inhibitors. The crystals diffract to at least 1.8 Å resolution and are suitable for X-ray structure analyses at high resolution.

2. Methods and results

2.1. Expression and purification

The full-length cDNA of mAMF (M14220) was amplified with sense (CGCCATGGCTG-CGCTCACC) and antisense (ATGGTGA-

GAAGGGACACG) primers and cloned into the *Eco*RI site of the pBR322 plasmid. *Eco*RI-digested mAMF cDNA was subcloned into the glutathione *S*-transferase (GST) gene-fusion plasmid pGEX-6P (Amersham) and transformed into *Escherichia coli* JM109 cells. Bacterial cultures were grown in LB medium (3 l shake flask with 1 l medium) at 298 K to an OD_{600} of 0.6. The expression of GST-mAMF was induced by 0.2 m*M* IPTG for 15 h at 298 K. After this period, cells were harvested by centrifugation at 8000*g* for 15 min and the cell pellet was frozen at 253 K. Cell-lysis buffer [150 m*M* sodium chloride, 1%(*v*/*v*) Triton X-100, 1%(*v*/*v*) NP-40, 1%(*w*/*v*) sodium deoxycholate and 10 m*M* EDTA in 20 m*M* Tris buffer pH 7.5] was added to the frozen cell pellets. Resuspended cells expressing fusion proteins were disrupted using ultrasonication on ice three times, each for 30 s. Cell lysate was clarified by centrifugation at 15 000*g* and applied to a 5 ml glutathione Sepharose FF column (Amersham) equilibrated with the cell-lysis buffer. Although the binding of GST-mAMF to the column may not be optimal in the presence of detergents, the use of detergents was essential to reduce non-specific binding of host proteins to the column. The column was washed with 20 column volumes of the cell-lysis buffer. After washing, the column was equilibrated with standard buffer (50 m*M* sodium chloride in 20 m*M* Tris buffer pH 7.5). The GST-mAMF bound to the column was digested by PreScission Protease (Amersham). After cleavage of the GST portion with the protease, fractions containing the GST-free mAMF were applied to another 1 ml glutathione Sepharose FF column to remove uncleaved GST-mAMF. The fractions containing mAMF were pooled and concentrated using a Centriplus-30 (Millipore) to 1 mg ml^{-1} and stored at 253 K after addition of glycerol to 20%(*v*/*v*).

2.2. Crystallization

A stock solution of 0.8 mg ml^{-1} mAMF with 40 m*M* sodium chloride and 20%(*v*/*v*) glycerol in 16 m*M* Tris buffer pH 7.5 was concentrated using a Centricon-30 to a protein concentration of 6 mg ml^{-1}. Crystallization was carried out at 293 K by the hanging-drop vapour-diffusion method. Crystallization conditions found by the sparse-matrix approach (Jancarik & Kim, 1991) were optimized to obtain larger crystals. In the best case, a droplet was prepared by mixing equal volumes (2.0 + 2.0 µl) of the protein solution (6 mg ml^{-1}) described above and reservoir solution [26%(*w*/*v*) polyethylene glycol 8000, 200 m*M* sodium acetate and 20%(*v*/*v*) glycerol in 100 m*M* cacodylate buffer pH 6.5] and was suspended over 500 µl reservoir solution. Plate-shaped crystals with typical dimensions of approximately 0.3 × 0.3 × 0.01 mm could be grown in two weeks (Fig. 1).

Crystals of the mAMF–inhibitor complexes were prepared as follows. The sodium salts of erythrose 4-phosphate (E4P), arabinose 5-phosphate (A5P) and 6-phosphogluconic acid (6PGA) were dissolved in the standard buffer described above to a concentration of 20 m*M*. The 6 mg ml^{-1} mAMF solution was mixed with aliquots of the respective inhibitor solutions at a volume ratio of 9:1 (final inhibitor concentration of 2 m*M*). The complex solutions were incubated for 30 min on ice prior to crystallization. Plate-shaped crystals having similar morphology to those of the inhibitor-free mAMF were obtained for each of the inhibitor complexes under the same conditions as for the inhibitor-free mAMF.

2.3. X-ray data collection

Since the crystallization conditions of mAMF described above contained 20%(*v*/*v*) glycerol in both the protein and reservoir solutions, X-ray data collection could be performed under cryogenic conditions without further addition of cryoprotectant. Thus, crystals in the hanging drop were directly mounted in nylon loops and flash-cooled in a cold nitrogen-gas stream at 100 K just prior to data collection. The data collection for inhibitor-free mAMF was performed by the rotation method at 100 K using an ADSC Q210 CCD detector with synchrotron radiation (λ = 1.00 Å at beamline NW12 of the Advanced Ring of the Photon Factory, Tsukuba, Japan). The Laue group and unit-cell parameters were determined using the *DPS* program package (Rossmann & van Beek, 1999). The Laue group was found to be 2/*m* and the unit-cell parameters were a = 69.97, b = 115.88, c = 73.27 Å, β = 101.76°. Only reflections with $k = 2n$ were observed along the (0*k*0) axis, indicating the monoclinic space group $P2_1$. Assuming the presence of two subunits (one dimer) per asymmetric unit led to an empirically acceptable V_M value of 2.32 Å^3 Da^{-1}, corresponding to a solvent content of 47% (Matthews, 1968). The current best diffraction data from an inhibitor-free mAMF crystal were collected to 1.8 Å resolution and processed with the program packages *DPS* and *CCP*4 (Collaborative Computational Project, Number 4, 1994) (Table 1).

Data from crystals of the inhibitor (E4P, A5P and 6PGA) complexes were collected by procedures similar to that described above. Crystals of the inhibitor complexes

Table 1
Data-collection statistics for mouse AMF.

Values in parentheses are for the outer resolution shell.

Data set	Free	E4P complex	A5P complex	6PGA complex
Space group	$P2_1$	$P2_1$	$P2_1$	$P2_1$
Unit-cell parameters				
a (Å)	69.97	69.63	69.93	69.72
b (Å)	115.88	115.86	115.88	115.57
c (Å)	73.27	73.32	73.32	73.26
β (°)	101.76	101.64	101.82	101.61
No. subunits per ASU	2	2	2	2
Solvent content (%)	47	47	47	47
X-ray source	PF-AR NW12	PF BL6A	SPring-8 BL41XU	PF BL6A
Detector	ADSC Q210	ADSC Q4R	MAR 165	ADSC Q4R
Wavelength (Å)	1.000	0.978	1.000	0.978
Resolution (Å)	1.80 (1.90–1.80)	1.80 (1.90–1.80)	1.65 (1.74–1.65)	1.70 (1.79–1.70)
Unique reflections	103572	105525	135679	124094
Multiplicity	3.0 (3.1)	3.4 (3.4)	3.4 (3.4)	3.5 (3.3)
Mean $I/\sigma(I)$	5.3 (2.1)	7.4 (2.0)	8.9 (2.1)	7.2 (2.2)
B factor (Wilson plot) (Å^2)	16.6	16.0	15.9	16.1
R_{sym} (%)	9.8 (33.6)	8.5 (37.3)	6.8 (36.1)	7.9 (33.3)
Completeness (%)	98.1 (99.7)	100 (100)	99.0 (98.3)	99.6 (99.7)

Figure 1
Monoclinic crystals of inhibitor-free mAMF.

were isomorphous with those of the inhibitor-free crystals. Data-collection statistics are summarized in Table 1. The relatively high R_{sym} values appear to be a consequence of radiation damage to the thin mAMF crystals used for data collection.

2.4. Crystallographic analyses

Initial phase determination for inhibitor-free mAMF was performed by the molecular-replacement (MR) technique with the coordinate set of inhibitor-free human AMF (PDB code 1jiq; Tanaka *et al.*, 2002), which has 90% amino-acid sequence identity to mAMF, as a search model. Water molecules were removed from the search model. Cross-rotation and translation functions were calculated using the program *AMoRe* (Navaza, 1994) from the *CCP*4 suite. The results showed clear initial solutions (correlation coefficient of 0.737 and *R* factor of 0.357 in the resolution range 15.0–2.5 Å) and a reasonable molecular arrangement of mAMF in the asymmetric unit (ASU). The model was improved by manual model building with the program *XtalView* (McRee, 1999) and tentatively refined to a resolution of 1.8 Å with an *R* factor of 0.222 (free *R* factor of 0.251), without incorporating water molecules, with the program *REFMAC* (Murshudov *et al.*, 1997). The refinement of the inhibitor-free mAMF at 1.8 Å is incomplete at this time and work on it continues.

The incompletely refined inhibitor-free mAMF structure could nevertheless be used for structure determination of the three (E4P, A5P and 6PGA) inhibitor complexes by the difference Fourier method. The amplitude (F_{calc}) and the phase angles calculated from such a partial structure (without bound inhibitor molecules) were then used to calculate $F_o - F_c$ difference Fourier maps. The residual densities clearly showed bound inhibitor molecules for all of three complexes. Model building and refinement of the inhibitor molecules in these maps are in progress.

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