Crystallization and Preliminary Crystallographic Analysis of Allograft Inflammatory Factor 1

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Abstract: Allograft inflammatory factor-1 (AIF-1) is a 17-kDa IFN-γ inducible Ca\textsuperscript{2+}-binding EF-hand protein involved in immune dysfunction and smooth muscle cell activation. AIF-1 was solubly expressed in \textit{E. coli} and purified. Crystals of AIF-1 were grown at 291 K using PEG-8000 as precipitant. Diffraction by the AIF-1 crystal extends to 3.3 Å resolution, and the crystal belongs to the space group P4\textsubscript{1} with unit cell parameters a=b=73.4, c=49.1 Å.

Keywords: Allograft inflammatory factor 1, prokaryotic expression, crystallization.

1. INTRODUCTION

Human allograft inflammatory factor-1 (AIF-1) is a 143-amino acid, cytoplasmic, IFN-γ inducible, Ca\textsuperscript{2+}-binding and EF-hand-containing protein. It is involved in immune dysfunction and smooth muscle cell activation. AIF-1 may participate in the progression of vascular proliferative disease on the basis of its ability to regulate the growth of vascular smooth muscle cells (VSMCs) [1-4]. Expression of AIF-1 increases markedly in monocytes and macrophages participating in allo- and autoimmune reactions, including perivascular inflammation in transplanted hearts, microglial infiltrates in experimental autoimmunity neuritis, and the inflamed pancreas of prediabetic BB rats [5,6]. In addition to its expression in mononuclear cells infiltrating cardiac allografts and microglia surrounding injured motor neurons, AIF-1 levels are increased in microglial cells in models of autoimmune encephalitis and in macrophages infiltrating the inflamed pancreas in a rat model of diabetes. Northern blot analysis revealed that AIF-1 is expressed in a variety of human tissues, with highest expression in tissues of lymphoid origin, in particular, spleen, blood lymphocytes, and thymus. Although its cellular function is currently unknown, AIF-1 may be important for monocyte/macrophage effector functions that contribute to inflammation.

AIF-1 was originally cloned and identified in rat cardiac allografts with chronic rejection states [7]. A similar study was carried out in transplanted human hearts [8]. It was also found that AIF-1 mRNA is present in injured rat carotid arteries [5]. Autieri (1996) cloned a full-length cDNA of AIF-1 from a human peripheral blood lymphocyte cDNA library. Sequence analysis demonstrated that human AIF-1 encodes a 143-amino acid polypeptide. Amino acid sequence motif analysis of AIF-1 revealed a consensus EF-hand helix loop domain that is a conserved feature of calcium-binding proteins [1]. The three-dimensional structure of the AIF-1 protein will help to further elucidate its biological functions. In this study, we report the expression of AIF-1 in \textit{E. coli} and its subsequent purification and crystallization. Crystals were obtained which diffract to 3.3 Å resolution.

2. METHODS

2.1 Primer Design and PCR Amplification

The primers were designed according to the published nucleotide sequence of AIF-1 (GenBank Accession No. U19713). In order to facilitate the subsequent cloning, two restriction endonuclease sites, \textit{BamH}I and \textit{SalI}, were attached to the 5’ terminal of the mutants.

The polymerase chain reaction was carried out using the human liver cDNA library as a template. The Taq polymerase and PCR amplification kits (including PCR buffer and dNTP mix) were obtained from Shanghai Sangon Co. (P. R. China). These samples were subjected to 35 cycles of denaturation (30s, 94 °C), annealing (30s, 55 °C), and extension (30s, 72 °C) using a DNA Thermal Cycler (Eppendorf).

The PCR product was separated on an agarose gel containing 1% agarose, purified by the DNA quick purify/recover kit (Beijing Dingguo Co., P. R. China) and digested with \textit{BamH}I and \textit{Sal}I overnight.

2.2 Cloning, Expression and Purification

The AIF-1 gene was cloned into the prokaryotic expression vector pGEK-6p-1 with \textit{BamH}I and \textit{Sal}I restriction enzyme sites, and the expected protein was expressed in \textit{Escherichia coli} BL21 (DE3) with high yield. The bacterial cells were resuspended in lysis buffer (1x PBS, 1 mM PMSF, 1 mM DTT) and homogenized by sonication. The lysate was centrifuged at 20,000 g for 30 min to remove cell debris. The supernatant was applied onto a GST-affinity column (1 ml glutathione Sepharose 4B) and the contaminant proteins were washed away with washing buffer (lysis buffer plus...
200 mM NaCl). The fusion protein was then digested with PreScission protease at 277 K overnight. The protein with an additional five amino acid tag (GPLGS) at the N-terminal was eluted with lysis buffer. The eluant was transferred to buffer A (25 mM Tris-HCl pH 8.0, 1 mM DTT) via a Sephadex G-25 column using the gel filtration chromatography method. The protein was concentrated using an Ultrafree 5,000 molecular-weight cutoff filter unit (Millipore) and further purified using a Superdex-75 (Pharmacia) column (using Buffer B: 10 mM Tris-Cl, pH 8.0, 100 mM NaCl, 1 mM DTT). The purified protein was analyzed on SDS-PAGE.

2.3 Crystallization

The purified protein was concentrated to about 20 mg ml⁻¹. Initial screening was performed at 291 K by the hanging-drop vapor-diffusion method using sparse-matrix screen kits [9] from Hampton Research (Crystal Screen reagent kits I and II), followed by a refinement of the conditions through the variation of precipitant, pH, protein concentration and additives. Typically, droplets of 4 µl were prepared on siliconized coverslips by mixing 2 µl of protein solution and 2 µl of the reservoir solution.

2.4 X-Ray Crystallographic Studies

X-ray diffraction data sets from AIF-1 crystals were collected in house on a Rigaku RU2000 rotating Cu anode X-ray generator at 48 kV and 98 mA (λ=1.5418 Å) with a Mar Research MAR345 image plate detector. The beam was focused by Osmic mirrors. The crystals were mounted in a nylon-fiber loop and flash-cooled in a liquid nitrogen stream at 100 K for a more detailed analysis. Crystals were immersed in a freezing solution for 5-10 s, picked up with a loop and then flash-cooled in a stream of liquid nitrogen cooled to 100 K. The reservoir solution was supplemented with 15% ethanol glycol used as a cryoprotectant. Indexing and integration of all images was performed in DENZO and scaling of the intensity data was performed in SCALEPACK; both are from the HKL program package [10]. The exposure time per frame was 8 s, the detector distance was 150 mm and the oscillation range per frame was 1°.

3. RESULTS AND DISCUSSION

3.1 PCR Amplification and Cloning of AIF-1

A 444 bp DNA fragment was obtained by PCR amplification (Fig. 1). The PCR product digested with BamHI and SalI was ligated with the pGEX-6P-1 vector digested with the same enzymes overnight at 16 °C. The BL21 (DE3) strain of E. coli was transformed using the ligation product. The bacteria containing the recombinant plasmids were identified and confirmed by sequencing.

3.2 Expression and Purification of AIF-1

AIF-1 fused with GST was solubly expressed in E. coli with a yield of about 5 mg ml⁻¹ culture. After a series of purification steps, the purified AIF-1 protein was > 95% pure on SDS-PAGE stained with Coomassie brilliant blue (Fig. 2). Dynamic light-scattering data showed that the protein had 70-80% homogeneity as a monomer.

3.3 Crystallization and Data Collection

Rod-like crystals appeared after about one month from one condition of Crystal Screen kit I (Hampton Research) containing PEG8000 as a precipitant (reagent 45 of kit I). The conditions were further optimized by variation of precipitants, buffer pH and protein concentration, and larger rod-like crystals (Fig. 3) were obtained. These larger crystals are reproducible and suitable for X-ray diffraction. The crystals were grown from a reservoir solution containing 18% PEG8000 in 100 mM sodium cacodylate buffer (pH 5.6) and 0.2 M Zinc acetate containing 1 M guanidine-HCl as an additive.

An X-ray diffraction data set was collected from a single AIF-1 crystal to 3.3 Å resolution (Fig. 4). The crystal be-
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longs to the space group \( P_4_3 \), with the unit cell parameters \( a=b=73.4, c=49.1 \) Å (see Table 1). It is estimated that there are two molecules in an asymmetric unit with a Matthews coefficient \( V_M \) of 2.06 Å\(^3\) Da\(^{-1}\) and a solvent content of 40%.

Table 1. Data Collection and Processing Statistics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
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<tr>
<td>Space Group</td>
<td>( P_4_3 )</td>
</tr>
<tr>
<td>Unit Cell parameters (Å)</td>
<td>( a=b=73.4, c=49.1 )</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
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<tr>
<td>Resolution (Å)</td>
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</tr>
<tr>
<td>No. reflections (observed)</td>
<td>20074</td>
</tr>
<tr>
<td>No. reflections (unique)</td>
<td>5867</td>
</tr>
<tr>
<td>Completeness (%)</td>
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</tr>
<tr>
<td>( \langle I/\sigma(I) \rangle )</td>
<td>3.1 (2.4)</td>
</tr>
<tr>
<td>( R_{int} ) (%)</td>
<td>20.0 (52.0)</td>
</tr>
</tbody>
</table>

Although we have obtained crystals of AIF-1, which diffract in-house to a resolution of 3.3 Å, we have so far been unable to determine the structure of AIF-1 by conventional methods. Work is underway to further optimize the crystallization and improve the resolution of diffraction beyond 3.3 Å. Since there are eight methionine residues in the AIF-1 molecule, the multiple-wavelength anomalous dispersion (MAD) method is considered to be the optimum method, and the preparation and crystallization of the selenomethionine derivative protein is currently in progress.

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