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Crystallization and preliminary crystallographic data for the augmenter of liver regeneration

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Abstract

A new cellular growth factor termed augmenter of liver regeneration (ALR) has been crystallized. ALR has been shown to have a proliferative effect on liver cells while at the same time producing an immunosuppressive effect on liver-resident natural killer cells and liver-resident mononuclear leukocytes. In addition, ALR appears to play an important role in the synthesis and stabilization of mitochondrial gene transcripts in actively regenerating cells. ALR crystals diffract to beyond 2 Å resolution and belong to space group $P2_12_12$, with $a = 125.1$, $b = 108.1$ and $c = 38.5$ Å. Based on four molecules per asymmetric unit, the Matthews coefficient is calculated to be 2.16 Å^3 Da^{-1} which corresponds to a solvent content of 43%.

1. Introduction

The mammalian liver is a unique organ in that it is capable of regeneration. For more than a century, investigators have been trying to understand the unique and complex process of hepatic regeneration and to identify the factors that are responsible for the liver's regenerative properties. Recently, a new heptatotropic factor called augmenter of liver regeneration (ALR) isolated (Fancavilla *et al.*, 1987) from the cytosol of weanling rat liver has been purified and cloned (Francavilla *et al.*, 1994; Hagiya *et al.*, 1994). Originally termed hepatic stimulatory substance (HSS) by LaBrecque (LaBrecque & Pesch, 1975), ALR has been shown to augment the liver regeneration that follows partial hepatectomy both in rats (LaBrecque & Pesch, 1975) and dogs (Terblanche *et al.*, 1980), and to augment the proliferation caused by portacaval shunt (Eck's fistula) in dogs (Starzl *et al.*, 1979). The proliferative response of ALR is however, limited to hyperplasic liver and no effect is observed on either resting liver, adult liver or cultured hepatocytes.

Although the exact mechanism of how ALR augments liver proliferation is at present unknown, ALR has been shown to reduce the activity of liver-resident natural killer cells (Francavilla *et al.*, 1997). This finding is consistent with the marked decrease in cytotoxic activity of liver-resident natural killer cells and mononuclear leukocytes following partial hepatectomy (Vujanovic *et al.*, 1995) and lends support to the idea (Burnett, 1971) of a strong relationship between the immune system and cellular proliferation.

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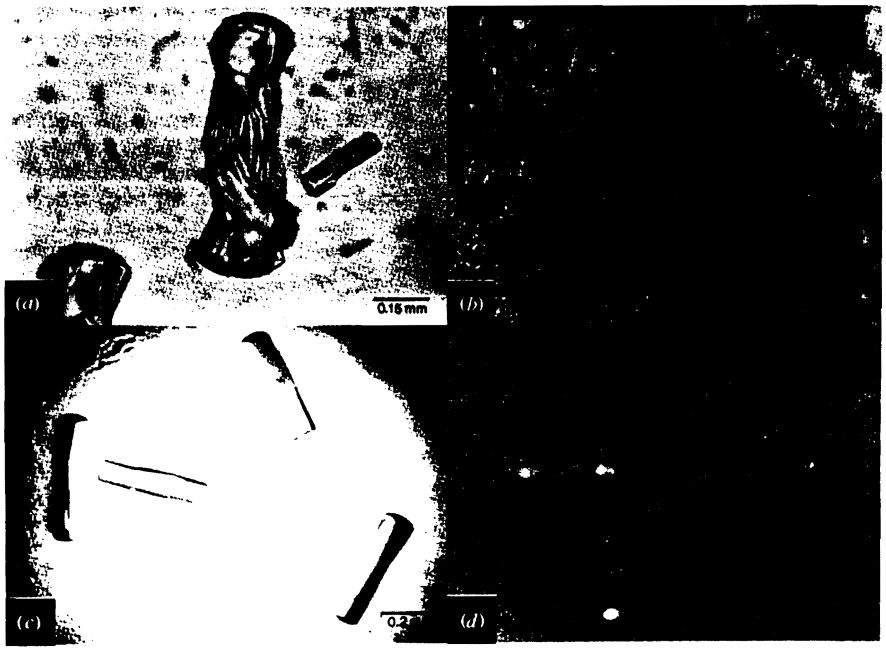

Fig. 1. Crystals of ALR-A: (*a*) ALR-A1 crystals grown at 301 K, (*b*) ALR-B1 crystals grown at 301 K, (*c*) ALR-A2 crystals grown by initial equilibration at 277 K followed by equilibration at 301 K, (*d*) ALR-A3 crystals grown by initial equilibration at 277 K and allowing the setup to slowly equilibrate to 301 K. The crystals are yellow in color. The red and green color of some of the crystals in the photographs is due to the effect of cross polarization on the plastic Linbro plate.

Interestingly, ALR is not restricted to the liver. An analysis of ALR mRNA expression in various rat tissues by Northern blot analysis (Hagiya *et al.*, 1994) showed tht ALR mRNA is expressed in relatively low abundance in almost all tissues studied with the exception of the liver and testis which showed a high level of ALR mRNA expression. This may suggest that ALR is not liver specific and that cells from other tissues may be capable of ALR expression under the appropriate conditions. The high level of ALR mRNA expression observed in the testis is in itself intriguing since the mouse *ALR* gene has been mapped to the T/t complex of chromosome 17 (Giorda *et al.*, 1996) which is involved in sperm function (Huw, Goldsboragh, Willison & Artzt, 1995; Silver, 1993).

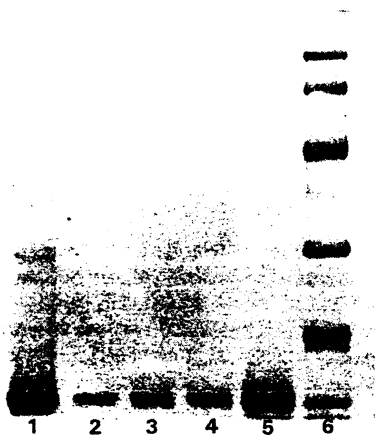

Fig. 2. The SDS–PAGE gels of the ALR: dissolved ALR-A2 crystals (lane 1), second wash (lane 2), first wash (lane 3), mother liquor from drop (lane 4), ALR concentrate used for crystallization (lane 5), and molecular weight markers (lane 6): phosphorylase B (top), 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31.5 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme 14.4 kDa (bottom).

Rat ALR has two related forms, a truncated or 'short' ALR (ALR125, $M_r = 15\,081$) which has 125 amino-acid residues and a 'long' ALR (ALR198, $M_r = 22\,800$) which contains 73 additional residues in the N-terminal segment of ALR. ALR198 is the result of an additional in-frame ATG initiation site which is 5′ to the initiation site used to code the 125-residue protein and represents the full-length protein (Giorda *et al.*, 1996). ALR 125 has been shown to be active *in vivo* using the Eck's fistula assay (Hagiya *et al.*, 1994).

The sequences of rat, mouse and human ALR are highly homologous (Giorda *et al.*, 1996) and show no homology to any known vertebrate protein. However, ALR does show a 50% sequence homology with the dual-function nuclear gene *ERV*1 (essential for respiration and viability) product (ERV1) from *Saccharomyces cerevisiae* (Lisowsky, 1992) which is part of the mitochondrial respiratory chain (Grivell, 1989) and

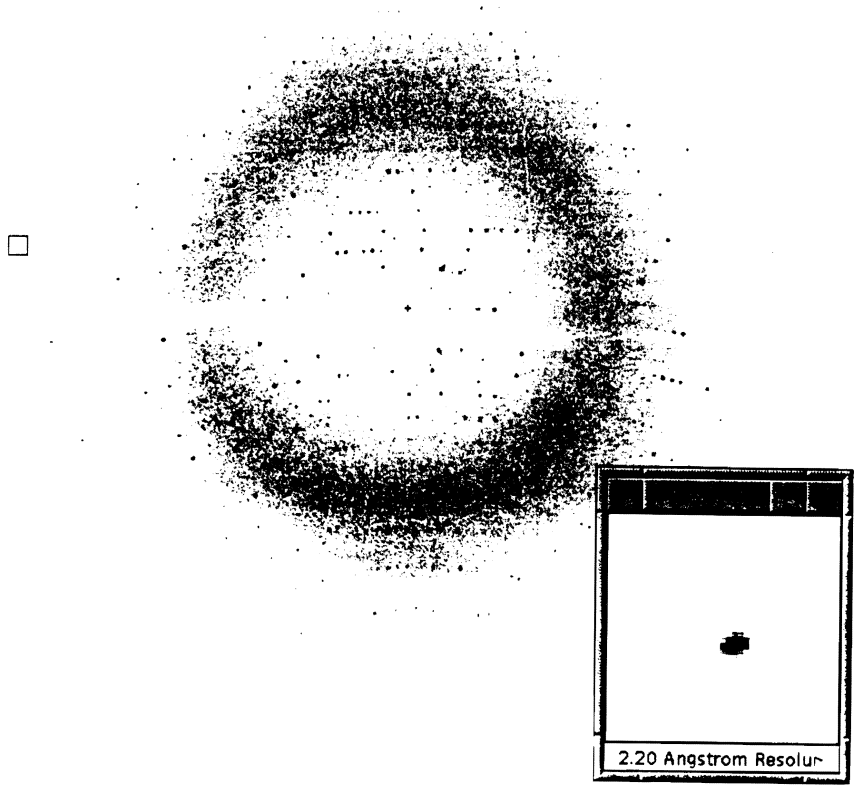

Fig. 3. A 1.0 oscillation photograph of ALR-A3 exposed for 1000 s. Data to 2.0 Å resolution were collected using a crystal-to-detector distance of 150 mm. The box shown indicates a reflection at 2.2 Å resolution.

plays an essential role in the control of mitochondrial gene expression (Lisowsky, 1994). Thus, ALR is thought to be the mammalian equivalent of ERV1 (Hagiya *et al.*, 1994) and codes for a component which plays an important role in both liver regeneration and the synthesis or stability of nuclear and mitochondrial transcipts (Giorda *et al.*, 1996). *ALR*125 has been cloned, expressed, purified and shown to be active *in vivo* using the Eck's fistula assay (Hagiya *et al.*, 1994). We report here the preliminary crystallographic data for crystals of ALR125.

2. Crystallization

The initial ALR125 sample (2 mg) was provided by the Pharmaceuticals Research Center, Toyobo Co. Ltd, Ohtsu, Shiga, Japan. Details of the isolation and purification are described by Hagiya *et al.* (Hagiya *et al.*, 1994). The sample was checked for purity using SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and divided into two equal fractions. The first fraction ($\simeq$ 1 mg) was concentrated to 8 mg ml^{-1} by centrifugation using Centricon tubes at 7500 rev min^{-1} and used for the first crystallization screen. In this screen, 94 crystallization conditions were analyzed using the Hampton Crystal Screen which is based on sparse-matrix sampling method of Jancarik & Kim (Jancarik & Kim, 1991). The screen was carried out at 301 K using 2 µl hanging drops (McPherson, 1982) set up in Linbro plates.

From the first screening two crystal forms were obtained. The form *A* crystals (ALR-A1, Fig. 1*a*) were grown from droplets containing equal volumes of the protein concentrate and a reservoir solution consisting of 0.1 *M* Tris buffer pH 8.5, 20% polyethylene glycol 2000 monomethyl ester (PEGMME 2000) and 0.01 *M* $NiCl_2$. These crystals grew over a period of 3–4 weeks to a size of approximately 0.15 × 0.15 × 0.5 mm. The crystals exhibited severe twinning and were judged unsuitable for X-ray diffraction studies.

The form *B* crystals (ALR-B1 Fig. 1*b*) were grown from droplets containing equal volumes of the protein concentrate and a reservoir solution consisting of 0.1 *M* MES buffer pH 6.5, 20% PEGMME 2000 and 0.01 *M* $NiCl_2$. These crystals were grown over a period of 2–3 weeks to a size of approximately 0.05 × 0.05 × 0.5 mm. These crystals were again judged unsuitable for X-ray diffraction studies because of their small cross-section.

The second ALR fraction ($\simeq$ 1 mg) was used to refine the crystallization conditions obtained for the form *A* (pH 8.5) crystals from the first screen. In order to determine if the twinning observed in the ALR-A1 crystals was a result of temperature effects, perhaps rapid nucleation, the crystallization experiment was repeated at 277 K. After a period of one week when no crystals were observed the setup was transferred to a 301 K incubator where crystals (ALRA-2, Fig. 1*c*) were observed after a few days and grew to dimensions of 0.10 × 0.10 × 0.3 mm in a period of 2–3 weeks. Although the quality of the ALR-A2 crystals was much improved these crystals still appeared to be twinned. A third experiment was then carried out in which the crystallization setup was first equilibrated at 277 K for 1 week at which time the setup was then placed in a double-insulated Styrofoam box and transferred to a 301 K incubator where it was allowed to slowly equilibrate to 301 K over a period of 1–2 weeks. The crystals (ALR-A3, Fig. 1*d*) grown by this procedure appear to be single under polarized light and can be reproducibly grown to a size of 0.25 × 0.20 × 0.60 mm.

SDS-PAGE was used to analyze the components of crystals. For this analysis, several small ALR-A2 crystals were washed with 300 µl of protein-free mother liquor, spun down in a centrifuge and the supernatant removed. The washing procedure was then repeated three times. The washed crystals were dissolved in sample-treating buffer [0.125 *M* Tris–HCl, 4% SDS, 20%(*v*/*v*) glycerol, 0.2 *M* dithiothreitol, 0.02% bromophenol blue, pH 6.8] and loaded onto an SDS gel. Other lanes of the gel were loaded with ALR concentrate and molecular weight markers (Fig. 2). After electrophoresis, the gel was stained with Coomassie Brilliant Blue and revealed only a single stained protein band. The mobility of the protein band from the SDS gel was established by comparison with known protein standards. It indicated that the dissolved crystals contained a protein with a molecular weight of approximately 15–16 kDa which agrees well with the molecular weight of 15 kDa (125 amino acids) for ALR.

3. Space group determination and data collection

For X-ray analysis, a crystal of ALR-A3 measuring 0.25 × 0.20 × 0.60 mm was mounted in a thin-walled glass capillary which contained a small amount of mother liquor to prevent dehydration. A data set to 2.0 Å resolution consisting of 110 1.0° oscillation images was collected on a MAR Research 30 cm image-plate detector using a crystal-to-detector distance of 150 mm. Each image was exposed for 1000 s with mirror focused (Yale design) 5.0 kW Cu $K\alpha$ X-rays. The data were indexed, integrated and scaled using *HKL* (Minor, 1993; Otwinowski, 1993).

The X-ray data set contained 146 155 measurements, 32 875 unique reflection (88.5% complete to 2.0 Å resolution), had an R_{symm} of 0.089 and indexed in a primitive orthorhombic lattice with cell constants a = 125.1, b = 108.1 and c = 38.5 Å. A representative data frame is shown in Fig. 3. Analysis of the three-dimensional diffraction data [*XPREP* (Sheldrick, 1991)] indicated that the space group was $P2_12_12$. Based on four molecules per asymmetric unit, the Matthews coefficient (Matthews, 1968) is calculated to be 2.16 Å^3 Da^{-1} which corresponds to a solvent content of 43%. Since non-reducing SDS-PAGE suggested that ALR may associate as homodimers (Hagiya *et al.*, 1994), a self-rotation function (Rossmann & Blow, 1962) analysis was carried out. The following programs were used in the analysis: *MERLOT* (Fitzgerald, 1988), *X-PLOR* (Brünger, 1990) and *AMoRe* (Navaza, 1994). However, the self-rotation analysis was inconclusive since no large non-origin peaks were observed on the κ = 90, 120 or 180° sections. The structure determination using isomorphous replacement is under way.

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