Crystallization of Porcine Liver Ribonuclease Inhibitor a Member of the Family of Proteins Containing Leucine-rich Repeats

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Single crystals of the RNase inhibitor from porcine liver have been obtained from 30 to 34 % saturated ammonium sulphate solutions at pH 6.0 to 7.2, containing 20 mM dithiothreitol, at room temperature over a period of two to three weeks. Because the inhibitor contains 30 1/2-cystinyl residues, all of which occur in the free thiol form, crystallization experiments were carried out in a desiccator under a nitrogen atmosphere. The crystals belong to the tetragonal space group *I*4, with cell dimensions $a = b = 134.76$ Å and $c = 83.65$ Å. The asymmetric part of the unit cell contains two molecules with a molecular mass of 49,093 Da, as could be shown with a self-rotation function calculated in the resolution range 10.0 to 3.2 Å. The crystals diffract to at least 3.2 Å resolution and are suitable for an X-ray structure determination.

Keywords: crystallization; ribonuclease inhibitor; leucine-rich repeat; X-ray diffraction

Neutral ribonuclease (RNase) activity in mammalian tissues is found in an inactive state due to complex formation with a cytoplasmic, proteinaceous inhibitor (RI, RNase Inhibitor†) (Pirotte & Desreux, 1952; Blackburn & Moore, 1982). The exact biological function of RI is not known with certainty, but it has been observed that the ratio of RI to RNase activity is high in tissues that are active in protein synthesis and in proliferating cells. In contrast, this ratio is low in catabolically active tissues. This led to the hypothesis that RI is involved in the regulation of intracellular RNase activity and, therefore, of RNA metabolism (Kraft & Shortman, 1970).

RI inhibits bovine pancreatic RNase A by forming a reversible one-to-one complex with a K_d of 67×10^{-15} M (Vicentini *et al.*, 1990). This high affinity results from a nearly diffusion-limited association rate constant ($k_{on} = 1.5 \times 10^8$ M⁻¹ s⁻¹) and a very low dissociation rate constant ($k_{off} = 8.3 \times 10^{-6}$ s⁻¹) (Vicentini *et al.*, 1990). Kinetic

analysis suggests that complex formation occurs *via* a two-step mechanism (Vicentini *et al.*, 1990; Lee *et al.*, 1989a). Initially, a weak complex is formed that does not involve the active site of the enzyme, followed by the formation of the tight complex. Substrate only competes in the second step. Interestingly, the two catalytic site histidyl residues (His12 and His119) are not involved in inhibitor binding, but a major interaction involves Lys41 (Blackburn & Jailkhani, 1979; Blackburn & Gavilanes, 1980), the residue that stabilizes the pentaco-ordinated transition state during RNA cleavage (Alber *et al.*, 1983). Also non-pancreatic, but homologous, RNases (Beintema *et al.*, 1988) are inhibited by RI. For instance, angiogenin, a homologue that induces blood vessel outgrowth, binds tightly to the human inhibitor ($K_d = 0.7 \times 10^{-15}$ M) (Lee *et al.*, 1989a,b), which abolishes both its ribonucleolytic and angiogenic activity (Shapiro & Vallee, 1987).

The amino acid sequence of porcine liver RI (Hofsteenge *et al.*, 1988), as well as that of human placental (Lee *et al.*, 1988; Schneider *et al.*, 1988) and rat lung RI (Kawanomoto *et al.*, 1992) has been determined. It consists of a single *N*-acetylated polypeptide chain of 456 amino acid residues. The

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† Abbreviation used: RI, RNase inhibitor.

Figure 1. Schematic representation of the repetitive structure of ribonuclease inhibitor. Upper part: the repeating units, A and B, have been indicated. The shading indicates areas that can be removed without destroying the inhibitor activity. Lower part: consensus sequences of the A and B-type repeats of ribonuclease inhibitor. The 1-letter code has been used, and X indicates any amino acid, where a denotes an aliphatic amino acid. The part of the repeat that is strongly conserved in the entire family of leucine-rich repeats containing proteins has been underlined, and the conserved residues are shown bold.

molecular mass of the RNase inhibitor from porcine liver is 49,093 Da, as deduced from the amino acid sequence. The protein is built from 15 homologous repeats of 29 (A-type) or 28 (B-type) amino acids in length that occur alternately in the sequence (Fig. 1). Both types of repeats are characterized by a large number of leucyl residues at constant positions, but they can be distinguished by the specific residues at positions 11, 19, 22 and 26 of the repeat unit (Fig. 1). Not all 15 repeats appear to be necessary for RI activity. Deletion of residues 1 to 90 of porcine RI results in an inhibitor that has the same affinity for RNase A as the full length molecule (Hofsteenge *et al.*, 1991b), whereas removal of residues 140 to 253, or 311 to 367 from human RI results in K_d values that are in the pM range (Lee & Vallee, 1990a). (The numbering of porcine RI (Hofsteenge *et al.*, 1988) has been used; in comparison, human RI (Lee *et al.*, 1988) contains a four amino acid N-terminal extension.)

Another salient feature of the primary structure of porcine RI is the presence of 30 1/2-cystinyl residues, all of which occur in the free thiol form (Vicentini *et al.*, 1990). The activity of RI is dependent on the presence of reducing agents in the medium (Blackburn & Moore, 1982). In fact, treatment of the RI-RNase complex with reagents that block thiol groups causes inactivation of the inhibitor and release of the active enzyme (Blackburn *et al.*, 1977). Which of the 30 residues are critical for activity is unknown, but two reactive cysteinyl residues in the C-terminal part (Cys371 and Cys404) were identified by differential labeling to be at, or close to, the enzyme-binding site (Hofsteenge *et al.*, 1991a).

Together with 31 other leucine-rich repeats containing proteins, RI constitutes a superfamily of proteins with widely diverged functions. Amongst the members are found: enzyme inhibitors and activators (Hofsteenge *et al.*, 1988; Ohkura & Yanagida, 1991), peptide hormone receptors (McFarland *et al.*,

1989; Sprengel *et al.*, 1990), cellular adhesion molecules (Hashimoto *et al.*, 1988; Reinke *et al.*, 1988; Krantz & Zipursky, 1990), enzymes (Young *et al.*, 1989) and bacterial virulence factors (Leung *et al.*, 1991). For none of these proteins has the three-dimensional structure been determined. Comparison of the consensus sequences of these proteins reveals that the C-terminal sequence LXLXXNXL(X)₂₋₇L is the most constant part (L indicates leucine, but other hydrophobic amino acids also occur at these positions), whereas the N-terminal region is more variable (Fig. 1). The asparaginyl residue at position 19 of the consensus sequence occurs in 25 members of this family, whereas cysteine is present at this position in six proteins. Since RI contains both repeats in an alternating fashion (Fig. 1), it seems to be an ideal prototype for this family of proteins. The number of repeats in different members varies from one (Hickey *et al.*, 1989) to 41 (Reinke *et al.*, 1988), and no common denominator is apparent. Although most repeats are 24 amino acid residues long, shorter (Neame *et al.*, 1989) and longer versions (Schneider & Schweiger, 1991; Hofsteenge *et al.*, 1988) also occur. Moreover, the presence and length of non-repetitive insertions between individual repeats is different between proteins.

The function of the leucine-rich repeats in a number of proteins has been examined (Suzuki *et al.*, 1990; Colicelli *et al.*, 1990; Braun *et al.*, 1991; Lee & Vallee, 1990b; Hofsteenge *et al.*, 1991b). From these studies it has become apparent that most often, leucine-rich repeats are involved in protein-protein interactions, but that other interactions, like protein-membrane, are also possible (Reinke *et al.*, 1988). It has been proposed that the constant residues (in particular leucine) in the repeats form a common scaffold, with the more variable residues in between forming loops that determine the specificity for a particular (macromolecular) ligand (Hofsteenge *et al.*, 1991b; Schneider *et al.*, 1988).

Recombinant RI was purified as described previously (Vicentini *et al.*, 1990) and stored at -70°C . Protein solutions for crystallization were concentrated to 10 mg/ml, using an Amicon micro-concentrator, and dialyzed overnight at 4°C against 10 mM Tris·HCl, pH 7.5, containing 25 mM NaCl, 2 mM dithiothreitol (DTT), 1 mM EDTA and 0.1% PEG 6000 (w/v). Crystallization was performed by the hanging-drop method with drops consisting of 3 μl of protein solution and 3 μl of reservoir solution. Preparation and handling of the drops was carried out under a stream of nitrogen and the crystallization set-ups were stored in a desiccator, which was flushed with nitrogen at regular intervals. Initially, the sparse-matrix approach (Jancarik & Kim, 1991) was tried, but found to be unsuccessful. Neither were crystals obtained using poly(ethyleneglycol) of different molecular weights, or 2-methylpentanediol as precipitants. Crystals could be grown, however, with ammonium sulphate as the precipitant. Optimal conditions were established: 30 to 34% saturated ammonium sulphate, buffered with 5 to 15 mM Mes (2-(*N*-morpholino)-ethane-sulfonic acid) or Hepes (*N*-2-hydroxyethyl piperazine-*N'*-2-ethane-sulfonic acid) buffer, pH 6.0 to 7.2, containing 20 mM DTT, at room temperature. Under these conditions, a few weeks were required to grow crystals to a size of 0.2 mm $\times$ 0.2 mm $\times$ 0.5 mm. Mounting of the crystals was carried out using the same buffer from which they were grown, with a precipitant concentration that was 1 to 2% higher, however.

X-ray precession photographs showed that the crystals belong to the tetragonal space group 14. The unit cell parameters determined on a FAST area detector are $a = b = 134.76 \text{ \AA}$ and $c = 83.65 \text{ \AA}$. A self-rotation function showed the existence of a non-crystallographic 2-fold axis, located in a plane perpendicular to the crystallographic 4-fold axis, and making an angle of about 30° with the a -axis (height of self-rotation peak is 28.8 arbitrary units (3.5 sigma), compared with a height of 50.0 for the origin peak; 3000 reflections with the highest intensities between 10.0 and 3.2 $\text{\AA}$ were used). Thus, the crystal contains two protein molecules with a molecular mass of 49,093 Da per asymmetric unit. This gives a volume to molecular mass ratio (V_m) of $1.93 \text{ \AA}^3/\text{dalton}$, which is within the range observed for protein crystals (Matthews, 1977). Using a standard partial specific volume for globular proteins of $0.736 \text{ cm}^3/\text{g}$ (Smith, 1968), the estimated solvent content of the crystals is 37%. The crystals diffract to about 3.2 $\text{\AA}$ resolution as estimated from data collected with a FAST area detector on a rotating anode X-ray generator operated at 40 kV and 40 mA.

The three-dimensional structure of the RNase inhibitor from porcine liver is being determined by the isomorphous replacement method. The search for heavy atom derivatives is in progress.

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