PRELIMINARY X-RAY DIFFRACTION STUDIES ON AN ANTI-VIRAL PROTEIN

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An anti-viral enzyme from Phytolacca americana, known to inhibit protein synthesis has been crystallized in a form useful for high resolution x-ray diffraction studies. Cracking of crystals due to the introduction of heavy metals can be reduced by cross linking with glutaraldehyde using a rapid fixation method. Several apparent isomorphous heavy metal derivatives of the crystal have been found. The molecular weight of the protein has been reevaluated as 31,000 daltons.

The plant Phytolacca americana, commonly known as pokeweed, contains a protein capable of inhibiting the transmission or replication of a number of viruses. This protein, PAP[1], acts against plant viruses such as TMV (1) and against animal viruses such as polio (2) and influenza (3).

Cell free studies indicate that PAP's anti-viral activity results from its ability to disrupt protein synthesis. It has been shown to inhibit both globin and polyphenylalanine synthesis in reticulocyte ribosomal systems (4). The inhibition is characterized by a depression of EF-1 and an acceleration of EF-2 mediated GTP hydrolysis and the reduction of EF-1 mediated binding of aminoacyl tRNA. The elongation factors themselves are apparently unaffected by PAP, and it is the factor binding sites on the 60S ribosomal subunit which are altered (4). PAP can inhibit 98% of protein synthesis in an Artemia salina system, and the enzymatic nature of the inhibition is clearly shown by the

[1]Abbreviations: PAP, Phytolacca americana protein; PEG, polyethylene glycol; MeHg, methyl mercuric acetate

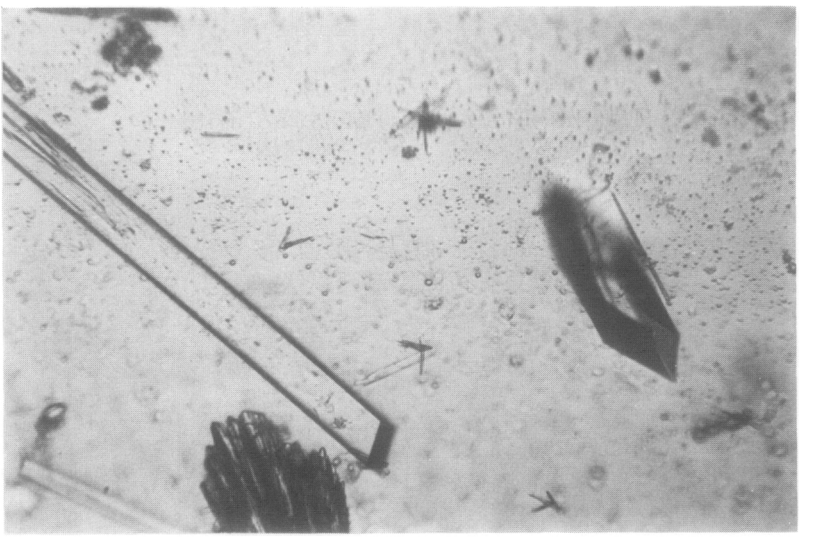

Figure 1: Crystals of PAP

observation that 50% inhibition results from a ribosome to PAP ratio of 150:1 (5). The enzyme cannot inhibit protein synthesis on pokeweed ribosomes, although it does inhibit all other plant systems tested (6). It is interesting to note that this mode of toxicity, inhibition of protein synthesis by disruption of the elongation factor activities, is similar to that used by the anti-tumor proteins Ricin (7) and RCA_{II} (8). The specific reaction involved in the toxic disruption by any of the proteins is as yet unknown. They may be specific nucleases or proteases or may carry out some more subtle reaction, such as a specific deamidation. It is hoped that analysis of the tertiary structure of PAP will help elucidate the mode of enzyme-ribosome recognition and the mechanism of this form of toxicity.

A method for isolating very pure PAP has recently been developed, and the protein was shown by gel filtration and SDS gel electrophoresis to be a single polypeptide chain of 27,000 daltons (5). We wish to report now that the molecular weight as determined by the Yphantis method (9) of sedimentation equilibrium is 31,000 daltons.

Dr. James Brown of the Clayton Foundation at the University of Texas has

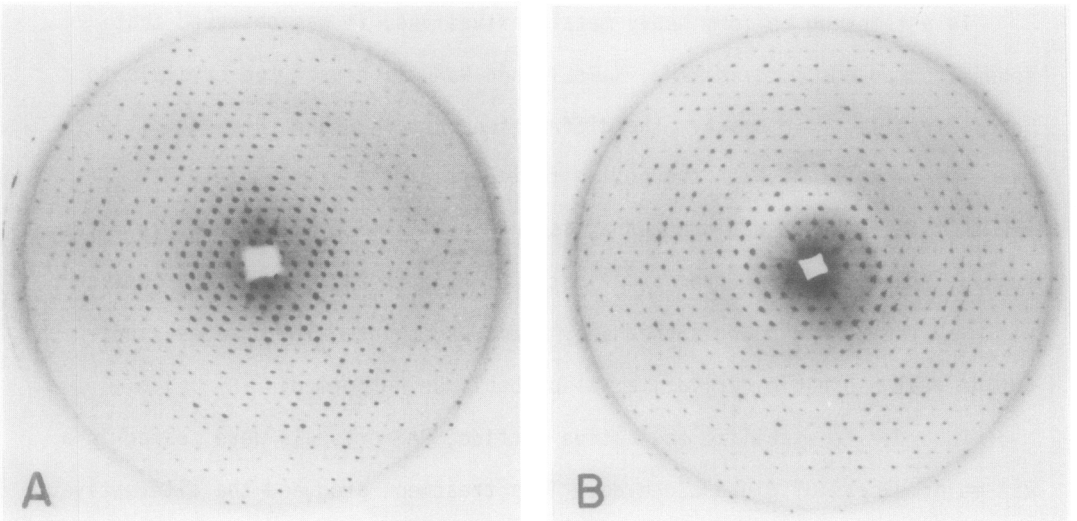

Figure 2: X-ray diffraction patterns taken at 16° precession angles. a) h0l zone, b) hk0 zone.

begun to determine the amino acid sequence of PAP. We hope that our projects will progress abreast of one another and that large parts of the linear sequence will be known when a three dimensional electron density map becomes available.

Crystals of PAP suitable for x-ray analysis are grown in 1 mm diameter tubes as follows. Three volumes of 40 mg/ml protein in 0.05 M Tris buffer, pH 7.4, are layered over two volumes of 50% (w/v) PEG (mw = 6,000). Crystals up to 1.0 x 0.4 x 0.2 mm (Figure 1) grow in 2-7 days at room temperature. The crystals are mechanically strong, diffract to at least 2Å resolution and show little intensity decay with exposure. The diffraction pattern (Figure 2) shows the crystal lattice to be triclinic space group P1, with a = 49.2Å, b = 49.0Å, c = 65.6Å, α = 80.6°, β = 112.9°, γ = 114.7° and cell volume = 132,200Å^3. We used a xylene and bromo benzene gradient with leucine and valine markers (10) to measure the density of a cross-linked PAP crystal as 1.20 g/cm^3. This is consistent with a protein mass of 63,500 daltons in the unit cell and indicates that two PAP molecules form the asymmetric unit and that the protein molecular weight is indeed close to 31,000. The V_m is 2.15, a typical value for protein crystals (11).

In attempting to form heavy metal derivatives, it was observed that several compounds such as MeHg, K_2PtCl_4 and $NaAuCl_4$ caused the crystals to become fragile or to crack. The diffraction pattern under these conditions was often consistent with the notion that part of the crystal retained the PAP lattice and part had adopted a distinct new lattice. In favorable cases, such as those resulting from a 1:1 ratio of MeHg to PAP, the native lattice was much the stronger and showed many isomorphous intensity changes, indicating that a useable derivative was probably being formed.

In order to stabilize the native lattice, PAP crystals were soaked in a 7:1 molar excess of glutaraldehyde. This treatment produced the alternative "sheared" lattice mentioned above, which we called SPAP. It is a strong crystal, diffracts to 2Å and has parameters a = 49.2, b = 52.5, c = 65.0, α = 77.8°, β = 11.5°, γ = 122.1° and volume = 132,400Å^3. SPAP is being considered for use as the parent crystal for three dimensional x-ray studies. Several compounds, such as mersalyl acid, produce some isomorphous intensity changes in the SPAP pattern, but in general this form appears less reactive than the native lattice and in particular the SPAP form cannot react with the rather specific sulfhydryl reagent MeHg.

A mechanically stable lattice, isomorphous with the native, and termed FPAP, was produced by dipping PAP crystals into a 30% PEG, 0.7% glutaraldehyde solution for two minutes and rinsing thoroughly with the 30% PEG pseudo-mother liquor. This operation apparently creates a cross-linked shell which maintains the physical integrity of the crystal but leaves the bulk of the interior cells in the native conformation. Several compounds, including K_2PtCl_4 and MeHg cause isomorphous intensity changes in the FPAP diffraction pattern. The suitability of these metals as derivatives is being assessed in projection prior to high resolution three dimensional data collection. Difference Pattersons were used to position both MeHg and $PtCl_4$ sites. Atomic positions and occupancies were then refined to produce model clusters. The MeHg derivative appears to have two major and one minor sites while the $PtCl_4$

derivative has four major sites. Phases calculated for the native from the Hg data were sufficient to return Pt positions in a difference fourier using $|F|_{Pt} - |F|_{Nat}$ as amplitudes. Similarly, phases calculated from Pt were sufficient to return Hg sites in a $|F|_{Hg} - |F|_{Nat}$ difference fourier. This is a very strong indication that these metals have phasing potential and can be used in three dimensions to solve the structure of FPAP.

Acknowledgements

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