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Characterization of Crystals of An Fab Fragment of a Murine Monoclonal Antibody

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The Fab fragment of an antibody, made against an E2-specific feline infectious peritonitis virus neutralizing antibody, has been crystallized in a form suitable for X-ray diffraction analysis from PEG 4000 using vapor diffusion methods. The Fab fragment crystals diffract to about 2.9 Å resolution and are of triclinic space group *P*1. Unit cell dimensions, by which the reciprocal lattice can be indexed, are $a = 57.16$ Å, $b = 70.85$ Å, $c = 75.81$ Å, $\alpha = 85.11^\circ$, $\beta = 121.28^\circ$ and $\gamma = 116.33^\circ$. There are two Fab fragments comprising the asymmetric unit of the crystals. The presence of a pseudo-mirror plane in the diffraction pattern suggests the presence of at least an approximate dyad axis relating the two Fab fragments within the asymmetric unit.

Keywords: crystals; Fab; X-ray; antibody

Feline infectious peritonitis virus (FIPV†) causes a fatal immune-mediated disease in cats for which conventional vaccine and therapeutic approaches have proven unsuccessful. Polyclonal antibodies to FIPV (Ab1) were generated by repeated immunizations of Balb/c mice with FIPV-infected *Felis catus* whole fetus cells and subsequently isolated and characterized as described elsewhere (Escobar *et al.*, 1991). From Ab1-secreting hybridomas, produced according to Goding (1980), a subset was isolated yielding monoclonal antibodies specific for the E2 peplomer of FIPV and possessing virus-neutralizing activity. These antibodies were conjugated to keyhole limpet hemocyanin (KLH) and hybridomas were generated from conjugate-immunized mice once the anti-Ab1 response was detected. Antibodies, including those whose crystallization we report here, were isolated and purified by ion exchange chromatography and characterized with regard to specificity by Western, and competitive Western, blot analyses.

The monoclonal antibodies raised against virus-neutralizing antibodies (Ab2) were shown to induce virus cross-reactive antibodies (Ab3) and to evoke substantial levels of protection against FIPV in seronegative cats (Escobar *et al.*, 1991). Subsequent studies are being carried out to characterize Ab2 to determine the extent of antigenic mimicry. In order to better understand the significance of this homology, we attempted to grow crystals of the protein on which to carry out a diffraction analysis that would allow us to delineate the three-dimensional structure of the homologous regions.

Fab fragments were prepared by papain digestion using the AvidChrom F(ab) Kit (BioProbe International, Inc., Tustin, CA), according to manufacturer's instructions. One vial of papain was resuspended in digestion buffer and added to a 10 mg/ml solution of antibody. After a 15-minute digestion time, the papain was inhibited and subsequently complexed with anti-papain antibody. The mixture was then passed through a protein A cartridge which removed complexed papain, Fc fragments and undigested antibody. Fab fragments were collected in the flow-through fraction.

IEF analysis of the Fab fragments revealed the presence of one dominant band of approximate pI 6.2 and four minor isomeric variants. The Fab fraction was dialyzed against 10 mM-Tris·HCl buffer (pH 7.0) containing 20 mM-NaCl (buffer A). Fab

† Abbreviations used: FIPV, feline infectious peritonitis virus; Ab1, polyclonal antibodies to FIPV; KLH, keyhole limpet hemocyanin; Ab2, monoclonal antibodies raised against virus-neutralizing antibodies; Ab3, virus cross-reactive antibodies; IEF, immunoelectrophoresis; f.p.l.c., fast protein liquid chromatography; PEG 4000, polyethylene glycol 4000.

isomers were bound to anion exchange resin (Mono Q HR 10/10 column, Pharmacia) and separated by f.p.l.c. with a continuous gradient of buffer A and buffer B (buffer A + 0.3 M-NaCl). The gradient consisted of a gradual increase of buffer B from 10% to 15% (v/v) over a period of 35 minutes. The resulting chromatograph clearly showed resolution of one major and four minor peaks corresponding to the Fab isomers. Silver-stained IEF analysis of the resulting fractions revealed the isolation of the dominant Fab isomer in a fraction free of extraneous proteins. This fraction was desalted against 10 mM-Tris · HCl + 25 mM-NaCl (pH 7.0) and concentrated to 12.5 mg/ml using an Amicon Centriprep 10. Retention of Fab binding activity was verified by competing HRP-labeled 409.5.3 whole immunoglobulin binding to Ab1 with unlabeled Fab fragments.

For crystallization experiments, a stock solution of the Fab fragment at a concentration of 15 mg/ml was used. Following the procedures described by McPherson (1990), a matrix of crystallization conditions was explored that included pH, precipitating agent, precipitant concentration and temperature. From these initial matrices, conditions were identified and then optimized that successfully and reproducibly yielded large crystals suitable for X-ray diffraction analysis.

The best crystals were obtained using the vapor diffusion method in Cryschem sitting-drop plates (Charles Supper Co., Natick, MA) using 20 μ l droplets and 0.75 ml reservoirs. The droplets were formed by mixing 7 μ l of the Fab fragment (15 mg/ml) with 3 μ l of a 0.1 M-acetate buffer at pH 3.6 to 4.6, and 10 μ l of the reservoir which was 16% to 20% PEG 4000 (w/v) in H₂O. The crystallizations were carried out at both 4°C and 22°C with the best crystals appearing at room temperature. The time required for crystal appearance was about five days with full growth occurring in about two weeks.

For preliminary analysis, crystals were sealed in quartz capillaries along with a small amount of mother liquor. Photographs of the reciprocal lattice were recorded using a Buerger precession camera with X-rays generated by an Enraf-Nonius generator fitted with a fine focus tube and operated at 45 kV and 32 mA. The radiation was nickel-filtered CuK α .

The faces of the crystals obtained as described above had more or less equal edge lengths, sometimes greater than 0.6 mm, and faces of dissimilar parallelograms. None of the angles between edges appeared to be 90°. An extensive investigation of the reciprocal lattice of several crystals (followed by similar explorations using a multiwire San Diego Area Detector system) demonstrated that no symmetry, other than that due to Friedel symmetry, was present in reciprocal space. The reflections could only be indexed on a triclinic lattice and the unit cell was, therefore, concluded to be of space group *P*1. Although it is possible to

index the diffraction intensities on other reciprocal lattices, the unit cell that we chose has $a = 57.16$ Å, $b = 70.85$ Å, $c = 75.81$ Å, (1 Å = 0.1 nm), $\alpha = 85.11^\circ$, $\beta = 121.28^\circ$ and $\gamma = 116.33^\circ$.

The volume of the unit cell is $V = 2.27 \times 10^{-5}$ Å³. If one assumes that there are two Fab fragments comprising the asymmetric unit, each of molecular mass about 50,000 daltons, then the volume to mass ratio for the crystal would be $V_m = 2.27$ Å³/dalton. This is near the center of the range found for most crystalline proteins (Matthews, 1968) and is most likely to be correct. Assumption of either one or three Fab fragments per asymmetric unit would result in a V_m of unusually high and low values, respectively.

An interesting feature of the diffraction pattern is the presence of an obvious pseudo-twofold axis perpendicular to the 100 plane of the crystal. This strongly suggests that a pseudo-dyad axis is present in real space and must, therefore, relate the two Fab fragments comprising the crystallographic asymmetric unit. Thus, we conclude that there is likely to be an exact or quasi 2-fold symmetry relationship between Fab fragments in these crystals.

The crystals diffract well to about 3.2 Å resolution with some data extending beyond that limit to perhaps 2.9 Å. The crystals appear to be very stable over many months of storage in their mother liquor, are mechanically stable and can withstand several days in the X-ray beam without unacceptable decay of their diffraction pattern.

The crystals described here are one of the few examples of Fab fragments from anti-idiotypic monoclonal antibodies and the structure solution will provide additional insight into their structural features and their mechanism of interaction. The determination of their structure will represent an important step in the successful application of immunological mimicry to the design of new classes of therapeutic agents (Wolff & McPherson, 1990).

The crystals are entirely suitable for a full three-dimensional structural analysis and this is currently being pursued using both molecular replacement and multiple isomorphous replacement methods.

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