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Crystallization and preliminary structure determination of an intact human immunoglobulin, b12: an antibody that broadly neutralizes primary isolates of HIV-1

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An intact human immunoglobulin with a full-length hinge has been crystallized for the first time in a form in which all of the Ig domains are ordered. The IgG1 antibody b12 is one of only three known monoclonal antibodies described that potently neutralize a broad range of HIV-1 primary isolates. It binds to an epitope overlapping the conserved CD4 binding site on the viral surface antigen gp120. Hexagonal crystals corresponding to space group $R32$ were grown from 0.8 M ammonium sulfate, with unit-cell parameters $a = b = 271.3$, $c = 175.2$ Å and one molecule per asymmetric unit. The crystals diffract to 2.8 Å and a preliminary molecular-replacement solution indicates that all 12 Ig domains of the antibody can be resolved.

1. Introduction

Antibodies are the key recognition molecules of the humoral immune system and serve as powerful adapter molecules that target foreign antigens to immunological effector systems for their elimination from the host. The variable Fab arms allow binding to a myriad of antigen shapes and sizes, while the constant Fc region interacts with effector systems such as complement and Fc receptors for IgG molecules. The Fab and Fc domains are connected by hinge regions of exceptional flexibility that facilitate changes in shape for bivalent attachment to antigen and for effector interaction (Burton, 1990). Electron microscopy of rabbit and mouse IgG has provided evidence for flexibility and arm rotation at the Fc–Fab and Fab–Fab interfaces, most of which is associated with the central hinge region (Valentine & Green, 1967; Green, 1969; Wrigley, Brown, Daniels et al., 1983; Wrigley, Brown & Skehel, 1983).

The inherent molecular flexibility has complicated crystallization of intact antibodies. While well over 200 structures of antibody fragments, mainly Fab and Fab′, have been determined, crystals of intact antibodies have only been reported eight times: Dob (Terry et al., 1968), Mcg (Edmundson et al., 1970), Kol (Palm & Colman, 1974), Zie (Ely et al., 1978), Mab231 (Larson et al., 1991; Harris et al., 1992), Mab4B7 (Stura et al., 1994), Mab24-404.1 and Mab61.1.3 (Harris et al., 1995). Only Dob, Mcg, Kol, Mab231 and Mab61.1.3 have yielded structures or partial structures.

Dob and Mcg are human myeloma proteins of the IgG1 subclass that contain natural hinge deletions. Short seven-residue hinge bypass peptides link the Fabs to the Fc; the constant regions of the light chains are linked by a disulfide not present in full-length IgG molecules. The hinge deletion and intra-light-chain disulfide bond restrict the movement of the Fabs and cause the Fc to abut tightly against them. As a consequence, these hinge-deleted antibodies are conformationally restricted and have reduced or no effector function (Klein et al., 1981). The crystal structure determinations of Dob and Mcg revealed compact symmetrical planar T shapes (Silverton et al., 1977; Rajan et al., 1983; Guddat et al., 1993).

Kol is also a human IgG1, but possesses the normal full-length hinge polypeptide. Crystals of this molecule only revealed density for the two Fab arms and the upper and core hinge regions (Colman et al., 1976; Marquart et al., 1980). No electron density could be attributed to the lower hinge or Fc region and it is likely that these regions are statically disordered. Similarly, a comparison of the diffraction patterns of the Zie IgG2 and F(ab)′₂ suggest that the Fc domain does not contribute significantly to the diffraction pattern (Ely et al., 1978). These early results suggested that for antibodies with normal hinges the relative placement of the Fc with respect to the Fab arms is not fixed and is free to assume a wide range of configurations.

Nevertheless, a well ordered antibody crystal can be obtained if a single conformer is incorporated into the crystal lattice owing to stabilization by crystal contacts. In this manner, a single 'snapshot' of an antibody structure can be obtained from the plethora of possible conformers. Crystals of two intact murine IgGs have previously been reported: Mab 231, a murine IgG2a directed against molecules on

the surface of canine lymphoma cells (Larson *et al.*, 1991; Harris *et al.*, 1992), and Mab 61.1.3, a murine IgG1 directed against the small molecule phenobarbital (Harris *et al.*, 1995, 1998). Their structures are both highly asymmetric and exhibit strikingly different conformations as a consequence of specific Fab–Fab and Fab–Fc contacts within the crystal lattice. Mab 231 displays a distorted T shape (172° between the long axes of the Fabs) and Mab 61.1.3 a distorted Y shape (115° between the Fabs). In both structures, the Fc domain is obliquely disposed relative to that of the Fabs (128° and 107°, respectively). These intact antibodies crystallize in the space groups $P1$ and $P2_1$, respectively, with one IgG per asymmetric unit. Thus, although the IgG is composed of two identical light and heavy chains, their conformers in the crystal are sufficiently asymmetric that at least one whole IgG molecule must comprise any asymmetric unit. On the other hand, the hinge-deleted Dob and Mcg, as well as the intact Kol in which the Fc is disordered, each crystallize with one-half of a molecule (one light chain and one heavy chain) in the asymmetric unit.

Here, we present the crystallization and preliminary structure analysis of b12, a human IgG1κ antibody that is a broad and potent neutralizer of HIV-1. This antibody was selected by phage-display technology from a bone-marrow library from a long-term asymptomatic HIV-1 infected male (Burton *et al.*, 1991) and is directed against a highly conserved epitope overlapping the CD4 binding site on the viral surface protein gp120. IgG1 b12 neutralizes a wide range of primary isolates of HIV-1 of different clades (Burton *et al.*, 1994; Trkola *et al.*, 1995; Kessler *et al.*, 1997; D'Souza *et al.*, 1997). Antibody b12 can bind to monomeric gp120 in solution or to oligomeric gp120 on the viral surface (Parren, Mondor *et al.*, 1998)
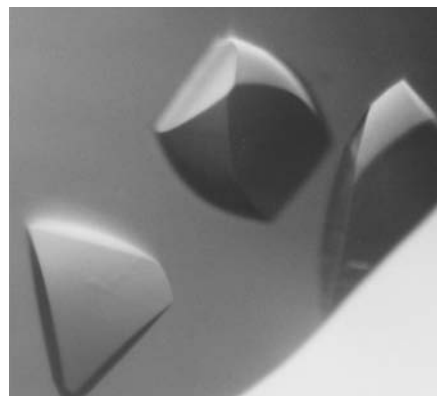
Figure 1
Crystals of the intact IgG1 b12.

and protects against infection with HIV-1 primary isolates in passive immunization experiments (Gauduin *et al.*, 1997). As this antibody is one of only three currently identified monoclonal antibodies that have this key combination of efficacy and broad specificity (D'Souza *et al.*, 1997), it may serve as a valuable template for HIV-1 vaccine design.

2. Methods and results

2.1. Protein purification

The Fab fragment of b12 was originally identified from a phage-display library (Burton *et al.*, 1991) and was converted into an entire IgG1 molecule for viral neutralization studies as described previously (Burton *et al.*, 1994). Recombinant IgG1 was expressed in Chinese hamster ovary (CHO-K1) cells in GMEM media containing 10% fetal calf serum (FCS). Standard protocols suggested weaning CHO cells from initial cultures in 10% FCS to 0.5% FCS before induction of human IgG expression in order to limit contamination with bovine IgG. However, numerous attempts to crystallize antibody (both whole IgG and its Fab cleavage product) produced in media containing 0.5% FCS were unsuccessful, as were attempts to crystallize recombinant Fab and scFv produced in *Escherichia coli*. However, higher FCS levels appear to foster increased cell and protein stability, as samples produced in 10% FCS were the only material that crystallized and protein yields in 10% FCS (20–33 mg l⁻¹) were at least 20-fold higher than those from identical cells weaned to 0.5% FCS (1 mg l⁻¹).

IgG is secreted into cell media and recovered using Protein A affinity chromatography followed by elution in 0.1 M citric acid, pH 3.0. Fractions were neutralized by 2 M Tris base pH 9.0 and dialysed against phosphate-buffered saline (PBS) pH 7.0. The resulting human immunoglobulin purity is greater than 99.6%, with less than 0.4% contaminating bovine IgG. All batches were tested for gp120 binding by ELISA and viral neutralization, as described by Parren, Wang *et al.* (1998). Existence of two full-length biantennary oligosaccharide chains on the Fc region was confirmed by hydrazine release and reacetylation using a GlycoPrep 1000 (Oxford Glycosciences, Abingdon, UK), 2-aminobenzamide labeling (Bigge *et al.*, 1995) and HPLC phase separation on a Glycosep-N chromatography column (Glyko Biomedical Ltd, Novato, CA, USA) at the Oxford Glycobiology Institute.

Carbohydrate structures were assigned according to Guile *et al.* (1996).

2.2. Crystallization

Initial crystallization conditions were identified with IgG at 16 mg ml⁻¹ in PBS pH 7.2 plus 0.02% sodium azide. A simple footprint screen (Stura *et al.*, 1992) produced small crystals in 3–7 d in 1 M ammonium sulfate, pH 5.5, by sitting-drop vapor diffusion. A decrease in ammonium sulfate concentration and a change in buffer to sodium cacodylate produced large crystals suitable for X-ray diffraction studies in 4–8 d. The b12 crystals reach full size (0.6 × 0.4 × 0.4 mm) after 2–4 d of growth and exhibit trapezoidal and pyramidal morphologies (Fig. 1). Final crystallization conditions are 800 mM ammonium sulfate, 100 mM sodium cacodylate, pH 6.5.

The successful use of a low-salt PEG 3350 and detergent screen (Harris *et al.*, 1995) was reported for the crystallization of three out of five intact murine monoclonal antibodies produced in hybridoma cell lines. However, the initial step in this crystallization procedure involves dialysis against ddH₂O for 24 h. That method was unsuitable for this study as IgG1 b12 is a euglobulin and is insoluble in plain water. Although Mcg, another euglobulin, crystallizes upon dialysis into low-salt solutions (Edmundson *et al.*, 1970), b12 irreversibly precipitates under these conditions. Kol and b12 both crystallize in ammonium sulfate (Kol in 1.5 M). Thus, low-salt screens may not be applicable to all IgGs.

2.3. Data collection and processing

Data were initially collected to 4 Å resolution at room temperature on a MAR Research imaging plate mounted on a Siemens rotating-anode generator in the laboratory (Siemens Inc., Madison, WI, USA) and on a Rigaku high-brilliance rotating-anode generator at University of California, San Diego (Rigaku Inc., The Woodlands, TX, USA). Subsequently, data were collected from single cryocooled crystals to 3.0 Å at beamline 5-2 of the Advanced Light Source (ALS) and to 2.8 Å resolution on beamline 7-1 at the Stanford Synchrotron Radiation Laboratory (SSRL). Crystals were mounted in loops (Hampton Research), dipped into mother liquor containing either 25% glycerol or 22.5–25% ethylene glycol as cryoprotectant and flash-cooled in liquid N₂. A 2.8 Å data set was collected at 96 K on a MAR Research imaging plate with a crystal-to-detector distance of 350 mm. Processing and scaling

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Space group	$R32$
Unit-cell parameters (Å)	$a = b = 271.3$, $c = 175.2$
Packing density V_M (Å^3 Da^{-1})	4.11
Solvent content (%)	69.8
Resolution (Å)	30–2.8 (2.85–2.80)
Total observations	170537 (8680)
Unique reflections	57121 (3179)
Redundancy	3.0 (2.7)
Average $I/\sigma(I)$	18.8 (2.4)
$R_{sym}(I)$ (%)	7.2 (51.3)
Completeness (%)	94.4 (95.3)

of the data were performed using the programs *DENZO* (Otwinowski, 1993) and *SCALEPACK* (Otwinowski & Minor, 1997) with an overall R_{merge} of 7.2% (see Table 1 for additional data statistics).

Crystals can be assigned to the space group $R32$ and were indexed in the triply primitive hexagonal unit cell with unit-cell parameters $a = b = 271.3$, $c = 175.2$ Å. As the Matthews coefficient of 4.11 Å^3 Da^{-1} and the corresponding 70% solvent content are fairly high, it was possible that two IgGs constituted the asymmetric unit (V_M of 2.05 and solvent content of 40%). The structure determination of IgG1 b12 proceeded by multi-body molecular replacement using *AMoRe* (Navaza, 1994) and individual Fab and Fc domains as search models. Although a molecular-replacement solution for an Fc domain was immediately clear, those of the Fabs were more elusive and nearly 100 search models had to be tested before solutions could be found. Very frequently, search models that produced one or two outstanding peaks in rotation functions yielded no solutions in translation functions. In fact, the combination of search model and rotation angles that ultimately succeeded in producing a clear translation solution for each of the Fabs yielded no clear solution in the rotation function. It seemed possible that an additional three solutions could be similarly masked in noise and it was not immediately apparent that the three identified solutions would be able to form a single IgG, given the arrangement and separation induced by the extreme asymmetry of the actual structure.

SDS gel electrophoresis of the crystals demonstrated that the hinge was indeed intact. Thus, the hinge region accounts for an additional 22 residues between the Fab and Fc search models, representing a maximal connecting distance of approximately 75 Å, if fully extended. Coordinates were calculated for one of the 18 Fcs in the unit cell and

then for each of the 18 copies of the first Fab solution, followed by each of the 18 copies of the second Fab solution. Only one combination of two Fabs and one Fc yielded distances of less than 75 Å from the C-terminus of the Fab heavy-chain globular domain to the N-terminus of the Fc model (34 Å for one Fab and 44 Å for the other). Thus, in the structure determination, only one 151 kDa IgG molecule is found in the asymmetric unit, with a Matthews coefficient V_M (Matthews, 1968) of 4.11 Å^3 Da^{-1} and a solvent content of 69.8%.

The R value after molecular replacement was 0.48 and R_{cryst} and R_{free} after rigid-body refinement were 0.45 and 0.47, respectively. The successful search model used for the two Fab domains displays a mere 52% sequence identity to b12 (PDB entry 1cbv; Herron *et al.*, 1991). Upon correction of the model sequence to the b12 sequence, R_{cryst} and R_{free} were reduced to 0.37 and 0.44, respectively. Model building and refinement are in progress to 2.8 Å resolution. However, visual inspection of the initial maps has already revealed interpretable electron density for all Ig domains and the carbohydrate. Density for the flexible hinge region is being improved through density modification and phase weighting and recombination.

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