FOR THE RECORD

Preliminary crystallographic studies of an anti-HIV-1 protease antibody that inhibits enzyme activity

JULIEN LESCAR,¹ RENATA STOURACOVA,^{1,2} MARIE-MADELEINE RIOTTOT,¹
VÉRONIQUE CHITARRA,¹ JIRI BRYNDA,^{1,2} MILAN FABRY,² MAGDA HOREJSI,²
JURAJ SEDLACEK,² AND GRAHAM A. BENTLEY¹

¹ Unité d'Immunologie Structurale, Institut Pasteur, Paris, France

² Department of Gene Manipulation, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czechoslovakia

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Abstract: F11.2.32, a monoclonal antibody directed against the HIV-1 protease, displays strong inhibitory effects toward the catalytic activity of the enzyme. The antibody cross-reacts with peptides 36–46 and 36–57 from the protease. Crystals of the Fab have been obtained both in the free state and as complexes formed with the protease peptide fragments, 36–46 and 36–57. Diffraction data have been collected for the free and complexed forms of Fab F11.2.32 and preliminary models for the crystal structures were obtained by molecular replacement.

Keywords: cross-reactivity; crystal structure; enzyme inhibition; Fab fragment; HIV-1 protease

The HIV-1 genome encodes a homodimeric aspartate protease (HIV-1 PR) that specifically cleaves the viral Gag and Gag/Pol polyprotein precursors (Katz & Skalka, 1994). A direct correlation has been established between the level of HIV-1 PR activity and particle infectivity (Rosé et al., 1995), and mutations abolishing proteolytic function have been shown to produce non-infectious virions (Kohl et al., 1988). Thus, precise regulation of protease activity is essential in the life cycle of the virus and much effort has been devoted to designing specific inhibitors of protease activity as possible antiviral agents (Wlodawer & Erickson, 1993). With a view to study both the function of HIV-1 PR and the eventual design of potential inhibitors of the enzyme, our interest has been directed toward the effects of anti-HIV-1 PR monoclonal antibodies on the catalytic activity of the protease. We describe here a preliminary crystallographic study of one of these mAbs, F11.2.32, which displays inhibitory effects on proteolysis by the enzyme.

Results and discussion: Recombinant wild-type HIV-1 PR was expressed in *Escherichia coli* as outlined previously (Ratner et al., 1985). The monoclonal anti-HIV-1 PR antibody, F11.2.32

(IgG1, κ), was obtained by the hybridoma technique of Köhler and Milstein (1975) by immunization of BALB/c mice with HIV-1 PR in the presence of Freund's adjuvant. The antibody was purified from ascites fluid as described for other immunoglobulins (Poljak, 1985) and the Fab was obtained by papain digestion. Both the IgG and Fab fragment of F11.2.32 show strong inhibitory effects on the catalytic activity of HIV-1 PR (J. Brynda, V. Chitarra, M. Fabry, M. Horejsi, J. Lescar, M.M. Riottot, R. Stouracova, G. Bentley, & J. Sedlacek, in prep.). The equilibrium dissociation constant for the binding of Fab F11.2.32 to HIV-1 PR was measured as 5 nM by surface plasmon resonance using the BIAcore system (Pharmacia Biosensor) (Johnsson et al., 1991).

Peptide segments 10–11 residues in length, and spanning the whole HIV-1 PR sequence, were tested previously for their ability to inhibit the binding of IgG F11.2.32 to HIV-1 PR (J. Brynda, V. Chitarra, M. Fabry, M. Horejsi, J. Lescar, M.M. Riottot, R. Stouracova, G. Bentley, & J. Sedlacek, in prep.). These experiments showed that, of all the peptide fragments assayed, only the peptide 36–46 competed with HIV-1 PR in binding to F11.2.32. We have performed additional measurements with peptide fragments 36–57 (containing the flap region) and 44–58. Peptide 36–57 showed similar competition to Fab–protease binding as peptide 36–46 (1 μ M of peptide gives approximately 50% inhibition in both cases), but peptide 44–58 was not recognized by F11.2.32, as expected from the earlier epitope mapping studies (J. Brynda, V. Chitarra, M. Fabry, M. Horejsi, J. Lescar, M.M. Riottot, R. Stouracova, G. Bentley, & J. Sedlacek, in prep.).

Although our main crystallographic objective in this study is to determine the structure of the FabF11.2.32–(HIV-1 PR) complex, the low stability of the protease has so far hindered progress in this direction. We have therefore crystallized Fab F11.2.32 as a complex with HIV-1 PR peptide fragments. Crystals of FabF11.2.32 in the unligated state and as complexes with peptides 36–46 and 36–57, were grown by vapor diffusion using the hanging drop technique. Crystals of free Fab F11.2.32 were obtained using a buffer of 20% PEG 4000 (w/v) and 0.1 M sodium citrate, pH 5.0. A volume of 2 μ L of Fab (13 mg/mL) was mixed

Reprint requests to: Graham A. Bentley, Unité d'Immunologie Structurale (CNRS URA 1961), Institut Pasteur, 25 rue du Dr. Roux, 75725 Paris; e-mail: bentley@pasteur.fr.

with 2 μ L of buffer and the resulting drop was suspended over a reservoir containing 1 mL of buffer. Crystals appeared after 5–7 days at 17 °C as thin plates with maximum dimensions of $0.40 \times 0.40 \times 0.05$ mm³.

To prepare the Fab–peptide complexes, the lyophilized peptides were resuspended in 10% (v/v) DMSO to yield a final concentration of 5 mg/mL. The Fab–peptide 36–57 complex was formed by incubating equal volumes of Fab F11.2.32 at 3.25 mg/mL and peptide at 1.25 mg/mL for 30 min at room temperature. This gives more than an eightfold excess of peptide over the Fab and is within the conditions that offer good competition in ELISA inhibition assays. Optimum crystallization conditions for the Fab–peptide 36–57 complex were found using a buffer composed of 24% PEG 2000, 0.04% NaN₃, 0.1 M Tris-HCl, pH 8.0, 150 mM NaCl. As with the unligated Fab, 2 μ L of protein solution were mixed with 2 μ L of buffer and the drops were suspended over a reservoir containing 1 mL of buffer. Under these conditions, crystals grew as prisms of up to $0.4 \times 0.4 \times 0.2$ mm³ in size in about 5 days at 17 °C. Crystals of the complex with peptide 36–46 were obtained in a similar manner. In this case, the buffer was 22% PEG 2000, 0.04% NaN₃, 0.1 M Tris-HCl, pH 8.0, and 2% (v/v) glycerol. Plate-shaped crystals appeared after about one week. The presence of bound peptide was verified by analyzing crystals, washed previously in buffer before dissolving, on a reverse-phase VYDAC C18 column (4.5×150 mm) using an acetonitrile linear gradient (0–50%) and monitoring the absorbance at a wavelength of 220 nm. Distinct peaks corresponding to the peptide and Fab were observed. The results of crystallographic data measurement for the three crystal forms, obtained in each case at 110 K after flash freezing, are given in Table 1. The complex FabF11.2.32–(peptide 36–57) has one molecule in the asymmetric unit, whereas the other two forms have two independent molecules.

Preliminary structural solutions for the three crystal forms were found by molecular replacement using the program AMoRe (Navaza, 1994). Because the elbow angle of the Fab F11.2.32 was an unknown parameter, independent search models for the variable and constant dimers, taken from entry 1baf in the Brookhaven Protein Data Bank (Bernstein et al., 1977), were used separately in the rotation and translation function calculations. For each crystal form, the first 20 peaks from the rotation function were analyzed systematically with the translation function. Clear and unambiguous solutions for both the free and complexed Fab fragments were obtained for all three crystal forms (see Table 2). Rotation and translation searches using the constant dimer returned the correct solutions as the highest peaks for all three crystal forms. For the rotation function of the variable dimer, on the other hand, the correct solution was the highest peak only for the complex with peptide 36–46. Nonetheless, a two-body translation search, where the constant dimer was held fixed (at the position indicated by the single-body search) and the variable dimer translated, unambiguously selected the correct orientation for the variable dimer in all three structures. The results thus obtained were entirely consistent with the single-body translation search with the variable dimer. For each crystal form, the independent treatment of the variable and constant dimers placed them in the correct stereochemical juxtaposition to form contiguous polypeptides for the light and heavy chains of the Fab. In addition, each solution placed the Fab in the unit cell without steric encumbrance with symmetry-related molecules in the unit cell. These two factors,

Table 1. Crystallographic data from measurements on crystals of FabF11.2.32, FabF11.2.32–(peptide 36–46) and FabF11.2.32–(peptide 36–57)^a

Crystal form	Space group and unit cell parameters	Resolution and R_{merge}	Completeness and redundancy of data measurements
FabF11.2.32	P2 ₁ $a = 68.9$ Å $b = 96.4$ Å $c = 70.6$ Å $\beta = 105.4^\circ$ $Z = 4$ $V_m = 2.2$ Å ³ /Da	2.9 Å 14.8%	90.9% 2.1
FabF11.2.32/ peptide 36–46	P2 ₁ 2 ₁ 2 ₁ $a = 82.4$ Å $b = 96.3$ Å $c = 105.8$ Å $Z = 8$ $V_m = 2.1$ Å ³ /Da	2.1 Å 11.3%	99.5% 4.7
FabF11.2.32/ peptide 36–57	P2 ₁ 2 ₁ 2 $a = 82.3$ Å $b = 97.7$ Å $c = 52.9$ Å $Z = 4$ $V_m = 2.2$ Å ³ /Da	2.6 Å 14.0%	99.7% 5.2

^a Intensity data were processed using the program DENZO and SCALEPACK (Otwinowski, 1993).

together with the high correlation coefficients obtained after rigid-body refinement (see Table 2), suggest that the solutions obtained are correct.

The two Fab–peptide complexes are very similar in the crystal packing of the molecules, as suggested by the unit cell parameters (see Table 1). Indeed, weak intensities observed at low resolution for odd l indices of the FabF11.2.32–(peptide 36–46) crystal form show that a noncrystallographic twofold rotation axis, relating the two independent molecules in the asymmetric unit, is located almost parallel to the c axis. Thus, the crystallographic twofold rotation axis in crystals of the peptide 36–57 complex (space group P2₁2₁2) becomes a noncrystallographic axis in crystals of the peptide 36–46 complex (space group P2₁2₁2₁) and the c cell length doubles. The orientations of the two independent molecules in the crystal structure of the complex FabF11.2.32–(peptide 36–46) were too close to each other to be resolved in the rotation function; the twofold symmetry of the molecular replacement solution was broken by alternately fixing one Fab molecule and refining the other as a rigid body until convergence was achieved. The elbow angles (the angles subtended by the approximate twofold axes relating V_H to V_L and C_H1 to C_L , respectively) are identical, within the limits of error, between all three crystalline forms.

For the two Fab–peptide complexes, sufficient volume (about 15 Å in diameter) was found above the antigen-binding site to accommodate the peptide. Electron density maps generated with phases from the models revealed density in these regions at more significant level than that observed in solvent regions elsewhere, possibly indicating the bound peptide.

Table 2. Results of molecular replacement for Fab11.2.32 in the free state and in the complexed state with peptides 36–46 and 36–57^a

	Fab (free)			
	Mol 1	Mol 2	Fab/36–46	Fab/36–57
Rv	0.093 (7) (0.110)	0.092 (10) (0.110)	0.121 (1) (0.110)	0.101 (5) (0.111)
Rc	0.125 (1) (0.110)	0.112 (2) (0.110)	0.127 (1) (0.087)	0.135 (1) (0.102)
Tv	0.131 (1) (0.127)	0.117 (3) (0.127)	0.120 (1) (0.113)	0.210 (1) (0.137)
Tc	0.190 (1) (0.067)	0.178 (1) (0.067)	0.123 (1) (0.067)	0.218 (1) (0.082)
Tcv	0.262 (0.177)	0.248 (0.177)	0.198 (0.152)	0.413 (0.210)
Rigid-body refinement	0.480 (<i>R</i> = 39.6%)		0.509 (<i>R</i> = 49.0%)	0.440 (<i>R</i> = 45.9%)

^a Variable and constant dimer searches with the rotation function (Rv and Rc, respectively) and the single-body translation function (Tv and Tc, respectively) give the peak height followed by its rank in parenthesis on the first line followed by the height of the largest noise peak in parenthesis on the second line. The row Tcv gives the result of the two-body search where the constant dimer was held fixed and the variable dimer was translated; the height of the largest noise peak is given in parenthesis on the second line. Results of the rigid-body refinement, where variable and constant dimers were refined independently, are given in the final row. All peak heights for the rotation and translation functions are given as correlation coefficients. Results of the rigid-body refinement are also given as the correlation coefficient with the crystallographic *R*-factor in parenthesis. Rotation function calculations were performed between the resolution limits of 20 and 4 Å, and translation function calculations and rigid-body refinements were performed between the resolution limits of 9 and 4 Å.

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