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A neutralizing antibody Fab–influenza haemagglutinin complex with an unprecedented 2:1 stoichiometry: characterization and crystallization

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The haemagglutinin HA is a trimer of identical subunits and is the more abundant viral surface glycoprotein of the influenza virus. It is the target of antibodies that neutralize viral infectivity. Antibodies that bind to HA with 3:1 and 1:1 stoichiometries have been identified. Here, an antibody whose Fab binds to HA with an unprecedented 2:1 Fab:HA stoichiometry is characterized. The complex has been crystallized and synchrotron data to 3.5 Å resolution have been collected. Molecular replacement confirms the stoichiometry of the complex.

1. Introduction

Haemagglutinin HA is the influenza virus glycoprotein that interacts with infectivity-neutralizing antibodies. As a consequence of this immune pressure, it is the virus component which is important in the antigenic drift that results in recurrent epidemics of influenza. HA has two roles in influenza infection: it binds the virus to its cellular receptors, sialic acid residues of glycoproteins or glycolipids, and following endocytosis it mediates the fusion of viral and cellular membranes to permit entry of the genome-transcriptase complex into the cell. HA is a trimer of identical subunits, each of which consists of two disulfide-linked polypeptides, HA1 and HA2. Structurally, each subunit consists of a membrane-proximal helix-rich stem structure and a membrane-distal receptor-binding globular domain (Wiley & Skehel, 1987).

The antigenicity of influenza isolates from outbreaks of disease and epidemics is regularly monitored to ensure the inclusion in vaccines of variants closely related to the circulating virus and this surveillance is accompanied by sequencing of the genes for the haemagglutinins of representative isolates (Fitch *et al.*, 1997). The antibody-binding sites on HA of viruses isolated since 1968 have been determined by locating sequence changes on the structure of the HA of A/Aichi/2/68 (X31); (Wiley *et al.*, 1981). It has been assumed that the sites of amino-acid substitution map to antibody-binding areas. The most direct evidence comes from the X-ray structure of the complexes of two neutralizing antibodies with HA, which show that all identified mutations that allow the virus to escape from neutralization by these antibodies are at positions located in their epitopes (Bizebard *et al.*, 1995; Fleury *et al.*, 1999).

Based on the comparison of the neutralization efficiencies of antibodies and Fabs binding to different antigenic sites on HA, the suggestion has been made that inhibition of receptor binding is an important component of their neutralization mechanism (Fleury *et al.*, 1999). The question remains as to how this inhibition is accomplished; the stoichiometry of the antibody–HA and Fab–HA complexes is an important component of the answer to this question. Evidence for 1:1 and 1:3 HA–antibody complexes has been obtained by estimating the molecular weight of these complexes (Poumbourios *et al.*, 1990). Electron microscopy has provided the clearest evidence for a 1:1 antibody–HA complex in the case of an antibody that selects a mutant HA changed at a residue near the trimer interface on the tip of the molecule, a site sufficiently large for only one antibody molecule to bind (Wrigley *et al.*, 1983). The distances of the locations on the HA trimeric structure of the mutants selected by various antibodies nevertheless suggest that a 3:1 antibody:HA stoichiometry is possible in many cases, with an antibody binding to each HA monomer and potentially blocking access to its receptor-binding site. Indications of such a stoichiometry come from electron microscopy of antibody–HA complexes (Wrigley *et al.*, 1983) and have been confirmed in two cases by the crystal structure of the corresponding 3:1 Fab–HA complexes (Fleury *et al.*, 1999, 2000). We report here the crystallization and characterization of an Fab–HA complex with a 2:1 stoichiometry, different from those previously observed. The properties of influenza virus variants escaping neutralization by the corresponding antibody have been described previously (Daniels *et al.*, 1987).

2. Methods

2.1. Purification

X31 virus and the bromelain-released ectodomain of X31 HA (BHA) were purified as described previously (Brand & Skehel, 1972). HC63, HC3 and HC21 antibodies and Fabs were produced and purified using standard methods. Briefly, HC21 and HC63 antibodies (both IgG1, κ) were purified from ascitic fluids by ammonium sulfate precipitation followed by protein A affinity chromatography (Pharmacia); conditions that achieve efficient binding of IgG1 (column equilibrated with 3 *M* NaCl, 1.5 *M* glycine–NaOH buffer pH 8.9) were used and elution was with 0.1 *M* citrate pH 6.0. HC3 antibody in 0.1 *M* Tris–HCl pH 8.0 was purified by affinity chromatograhy on protein A Sepharose; elution was with 0.1 *M* citrate pH 4.0. The Fab fragments were prepared by papain digestion in 0.1 *M* sodium phosphate pH 7.3, 1.25 m*M* EDTA, 1.5 m*M* 2-mercaptoethanol, using a 100:1(*w*/*w*) IgG to papain ratio; the digestion time was determined in a time course and stopped by addition of iodoacetamide to a 10 m*M* final concentration. The Fc fragments and the partially digested IgG were removed by chromatography on DEAE Trisacryl (IBF) in the case of HC63 and HC21 Fabs or on Protein A Sepharose (Pharmacia) in the case of HC3 Fab; in all cases, this was followed by gel filtration on Sephacryl 100HR (Pharmacia). The Fabs were incubated overnight with BHA to form a complex; there was a 30% Fab excess compared with the ratio of three Fabs to one BHA trimer. The excess Fab was removed by gel filtration. Complexes were analysed by FPLC with a Superose 12 HR column; 200 µl samples in 0.15 *M* NaCl were injected at a 0.4 ml min^{-1} flow rate and the elution volume was measured.

2.2. Analytical centrifugation

Sedimentation equilibrium experiments were carried out at 293 K on a Beckmann XL-A analytical centrifuge equipped with an An Ti 60 titanium four-hole rotor with two-channel 12 mm path-length centrepieces. 100 µl aliquots of 0.5 OD_{280} concentration were centrifuged at two rotor speeds (4500 and 5200 rev min^{-1}). Radial scans of the absorbance at 280 nm were taken at 3 h intervals and samples were judged to be at equilibrium by the absence of systematic deviations in overlaid successive scans. The partial specific volumes of the complexes were taken as 0.73 ml g^{-1} and the solvent density was calculated to be 1.0048 g ml^{-1}. Multiple data sets were analyzed by non-linear least-squares procedures provided with the Beckman Optima *XL-A* software package (McRorie & Voelker, 1993).

2.3. Crystallization and X-ray crystallography

The HC63–BHA complex was concentrated to a final absorbance (OD_{280}) of between 15 and 17 (given BHA absorbance and the average value of Fabs absorbance, this corresponds to a 10–15 mg µl^{-1} concentration, *i.e.* 33–50 µ*M*) and then crystallized by vapour diffusion in 24-well trays (Linbro) at 291 K. Hanging drops were composed of 2 µl HC63–BHA complex in 0.15 *M* NaCl, 0.04%(*w*/*v*) NaN_3 and 2 µl well solution. Trials were also performed by adding HC63 Fab to the purified concentrated HC63 Fab–X31 BHA complex such that the overall Fab:BHA ratio in the drop would be 3:1. All trials gave identical results; crystals grew from well solutions containing 13–15% PEG 20 000, 50 m*M* Tris–HCl pH 8.0 and 20 m*M* $CaCl_2$ and were harvested into the same solution containing 17% PEG 20 000 (all chemicals are from Merck). Crystals were prepared for cryocrystallography by transferring them to a harvest solution containing 20%(*v*/*v*) glycerol as a cryoprotectant. The crystals were picked up with a rayon loop, mounted on a magnetic cap and flash-cooled on the camera spindle at 100 K in a gaseous nitrogen stream (Rodgers, 1994).

Data collection was performed initially at the ESRF; data used in the final refinement were collected at the Elettra Synchrotron Source X-ray Diffraction beamline (λ = 1.00 Å) using a MAR 345 image-plate detector and a 425 mm crystal-to-plate distance. 0.6–0.2° oscillation photographs were collected. X-ray data were indexed and integrated using *MOSFLM* (Leslie, 1992) and were further analyzed with programs from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994).

Molecular-replacement calculations were performed with the *AMoRe* package (Navaza, 1994). The atomic coordinates of uncomplexed X31 BHA (PDB entry 1hgf), of HC45 Fab (PDB entry 1qfu) and of BH151 Fab (PDB entry 1eo8) were used as starting models; diffraction data used were between 15 and 4 Å resolution.

3. Results and discussion

HC63 Fab–X31 BHA crystals grew as thin plates, as shown in Fig. 1. The dimensions of the crystal used for the final data collection were 220 × 100 × 30 µm. SDS–PAGE demonstrated that the crystals contained Fab and HA polypeptide chains (data not shown). The space group was determined from the symmetry of the diffraction data set to be $P2_12_12$, with unit-cell parameters $a = 143.0, b = 315.6, c = 97.0$ Å, $\alpha = \beta = \gamma = 90°$. Crystals showed well ordered diffraction to 3.5 Å resolution, with a mosaicity of 0.6°. This precluded an oscillation range larger than 0.2° from being used for some orientations of the crystal, in order to avoid overlap between recorded reflections. The symmetry of the crystal implies that at least one HC63 Fab–X31 BHA complex is present in the asymmetric unit; with one complex in the asymmetric unit, the volume per dalton is between 3.9 Å^3 (in the case of one Fab per HA trimer) and 2.9 Å^3 (in the case of three Fabs per HA trimer), which is in the range of the values observed for proteins (Matthews, 1968).

Initial evidence for the stoichiometry of the HC63 Fab–HA complex came from gel-filtration and analytical centrifugation experiments. The complex elutes from a Superose 12 HR gel-filtration column at a volume of 9.3 ml, which is intermediate between the volumes observed in the case of complexes of X31 BHA with HC21 Fab (9.8 ml) and with HC3 Fab (8.7 ml), which respectively have a 1:1 and, presumably, a 3:1 Fab:HA stoichiometry (Wrigley *et al.*, 1983). Analytical centrifugation sedimentation equilibrium data for the HC63 Fab–X31 BHA complex were compared with those of complexes of the HC3 and HC21 Fabs. The data fit extremely well to monodisperse species with 261, 306 (Fig. 2) and 356 kDa masses for the HC21 Fab–BHA, HC63 Fab–BHA and HC3 Fab–BHA complexes, respectively. Given the mass of BHA (220 kDa) and that expected for a Fab (45 kDa), the purified complexes of BHA with HC21, HC63 and HC3 Fabs have 1:1, 1:2 and 1:3 stoichiometries, respectively; in the case of the HC63 Fab–BHA complex this

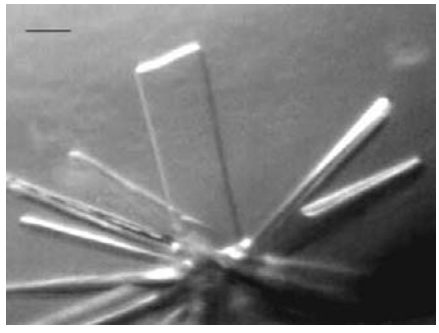

Figure 1
Crystals of the HC63 Fab–X31 BHA complex. The bar is 0.1 mm.

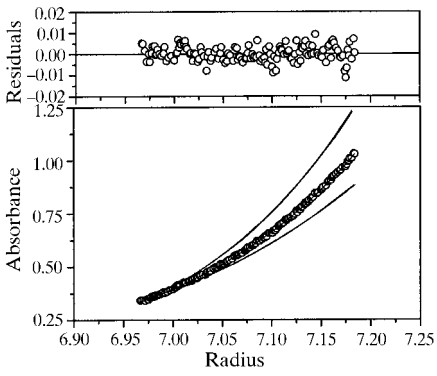

Figure 2
Fit of the X31 BHA–HC63 Fab equilibrium sedimentation data. Equilibrium sedimentation data obtained at 4500 and 5200 rev min^{-1} were fitted to a monodisperse model. The open circles represent the data from the 4500 rev min^{-1} run and the solid line is the fit. The residuals representing the variation between the experimental data and those generated by the fit are also plotted (upper panel); the molecular weight deduced from the fit is 306 kDa (standard deviation is 1 kDa). Theoretical curves for absorbance *versus* radius based on the molecular weights of the 1:1 and the 3:1 Fab–BHA complexes are also plotted (lower panel).

result is confirmed by a molecular-replacement study.

Rotation and translation functions were initially used to locate X31 BHA in the HC63 Fab–X31 BHA crystal. The result was unambiguous: after rigid-body refinement the correlation of observed and calculated structure factors was 0.51, the next highest coefficient for alternate models resulting from the molecular-replacement procedure being 0.38. The use of Fab variable domains or constant domains dimer models did not provide any solution to the molecular-replacement problem, probably because these models represent too low a proportion of the asymmetric unit content. We therefore reasoned that it might be possible to solve the molecular-replacement problem for the Fab by using whole Fab models. Given the well known variability of the elbow angles of these molecules (values between 127 and 227° have been reported; Wilson & Stanfield, 1994), models spanning the range of observed elbow-angle values were used. The best models for the crystal structure determination of the HC63 Fab–HA complex were not those which had their sequence closest to that of HC63 Fab, but those with elbow angles similar to those of HC63 Fabs in the crystal (HC63 Fab heavy-chain and light-chain sequences have been deposited with EMBL; accession numbers: AJ252270, AJ252271). The most contrasting solution was provided by the HC45 Fab (elbow angle: 144°): after rigid-body refinement the correlation of observed and calculated structure factors was 0.62, the next highest coefficient for alternate solutions resulting from the molecular-replacement procedure being 0.45. Starting from this structure a second Fab was located using the BH151 Fab as a model (elbow angle: 138°); in this case the correlation coefficient of observed and calculated structure factors was 0.73 after rigid-body refinement, the next highest coefficient for alternate solutions resulting from the molecular-replacement procedure being 0.55. On the basis of packing considerations, a third Fab molecule is allowed. However, no significant density was revealed for this third Fab in a σ_A-weighted map. Moreover, when incorporation of a third Fab in the model of the complex was attempted, the correlation coefficient between observed and calculated structure factors decreased for all the models that have been used.

Therefore, the analytical centrifugation characterization of the purified HC63 Fab–X31 BHA complex and the molecular-replacement study are consistent. The 2:1 stoichiometry HC63 Fab–X31 HA complex is the most stable one at the concentration used for analytical centrifugation (1.0 μM); our results suggest this is still the case when concentrations are in the 50 μM range, where crystallizations were performed. This contrasts with most HA specific Fabs which yield 1:1 or 3:1 complexes with BHA at these concentrations. Refinement of the structure should allow us to identify the interactions that make this complex more stable than the 1:1 and 3:1 Fab–HA complexes that are more commonly encountered.

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