Disulphide dimerised peptide creates a crystal contact in an anti-peptide antibody

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Although many Fab derived from human and murine antibodies have been crystallised, several such fragments, including anti-peptide Fab 4x11, remain problematic for crystallisation. Diffracting crystals have been obtained for this Fab only in complex with a cysteine containing peptide CGGIRGERA. The IRGERA portion contained in the C-terminus of histone H3 is recognised by the monoclonal antibody and the CGG portion is an essential spacer. Crystallisation experiments and seeding studies show that disulphide bond formation is essential for obtaining crystals. Data has been collected to 2.7 Å resolution, and the structure solved by molecular replacement. The asymmetric unit contains two antibodies whose binding sites are face-to-face in an non-crystallographic approximate two-fold rotational axis. We find two Fab in the asymmetric unit with the two antigen combining sites facing each other. This result was unexpected since it has been common practice to avoid the cysteine-containing peptides because of heterogeneity, since the peptide solution is likely to contain both dimeric and monomeric peptides. This result suggests that the cysteine containing peptides used in immunisation for coupling them to a carrier protein could also be used to screen for crystals. Structural data obtained for the same peptides as those used in the immunisation is valuable to evaluate to what extent the linker to the carrier protein may have contributed to the shaping of the antibody binding site. The introduction of exposed cysteines on the surface of proteins by site directed mutagenesis could help resolve difficult crystallisation cases. Should these not form a disulphide bridge in the crystal but nonetheless crystallise, the free cysteine could be used to make a heavy atom derivative for isomorphous replacement.

Keywords: protein engineering; anti-peptide antibody; modified peptides; heavy atom derivatives; disulphide bridge.

1. Introduction

Monoclonal antibody (mAb) 4x11, is one of a series of monoclonal antibodies raised against the model hexapeptide IRGERA, which corresponds to the COOH-terminal residues 130-135 of histone H3. These antibodies react strongly with the parent peptide, the cognate protein histone H3 and with nucleosomal particles. In addition they cross-react with the homologous retro-inverso analogues of the parent peptide and with reduced peptide-bond peptide analogues (Guichard *et al.*, 1994; Benkirane *et al.*, 1996). The aim of this study is to understand this cross-reactivity. We have prepared and purified Fab fragments of mAb 4x11, and set up crystallisation trials to obtain crystals of Fab alone and of Fab-peptide complexes with L-amino acid peptides as well as reduced bond peptides. Although in many cases Fab derived from human and murine antibodies can be easily crystallised alone and in complex with peptides, Fab 4x11 appears to be particularly problematic. To increase the chances of finding crystals we have screened all the available peptides in parallel using the modified Q-plate system (Stura, 2001). In the screen we have also included the cysteine containing peptides that were used in the immunisation. The cysteine is needed for coupling to a carrier protein, as the peptide is by itself non-immunogenic. In addition, to avoid that the epitope becomes buried within the carrier protein, a short flexible tail of two glycines is added between the cysteine and the recognised epitope. On the other hand, for crystallisation it is custom to synthesise peptides of different length without such appendices. Being able to screen a large number of complexes in parallel (Stura, 2001) it was possible to include in the crystallisation screen both the standard crystallisation-customised peptides and the cysteine-containing peptides. We report here that the cysteine containing peptide is the only one to yield X-ray quality crystals, and that the crystallisation is severely inhibited by insufficient dimeric peptide. Hence, the criticism that cysteine-containing peptides dimerise by disulphide bond formation and thus create a heterogeneous environment is valid. In our case the problem for crystallisation stems from an excess of the monomeric rather than the dimeric form of the peptide.

2. Materials and methods

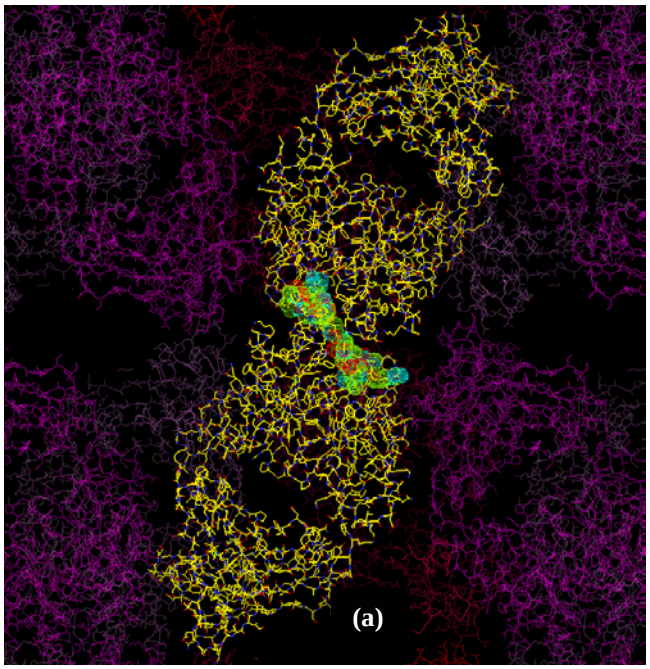
2.1. Production, sequencing and purification of Fab 4x11

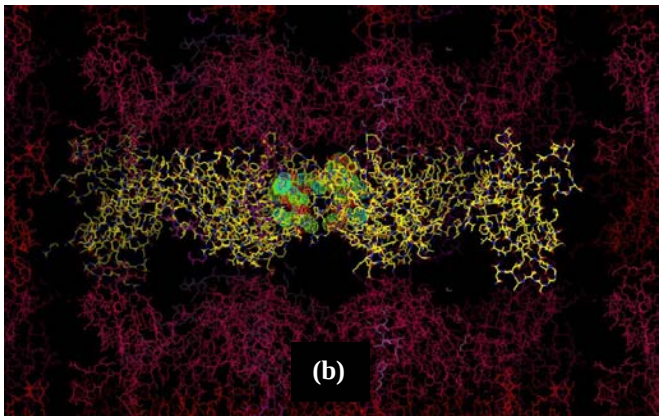
The production and characterisation of mAb 4x11 (IgG1, κ2), as well as the measurement in the BIAcore of its kinetic association and dissociation constants and its affinity constant with the peptide used for immunising mice, have been described previously (Benkirane *et al.*, 1995). Antibodies were obtained in large amounts from hybridoma injected intraperitoneally in nude mice pre-treated with Pristane (AgroBio, la Ferté St Aubin, France). Antibodies in ascites were purified on protein A hyper D (Biosepra), and their purity was estimated by SDS-PAGE to be ~95%. Fab (50 kDa) were obtained after cleavage of IgGs with papain (Sigma, St Louis, MA) at 37°C. The ratio of activated mercuripapain to IgG used was 1:20 (w/w) in phosphate buffered saline (PBS), pH 7.5 with 10 mM cysteine and 1 mM EDTA. The reaction was terminated by addition of 30 mM iodoacetamide. A preliminary pilot experiment was performed to determine the optimal conditions of digestion. After extensive dialysis against PBS pH 7.4, the Fab was purified on Sephacryl S-200 HR (Pharmacia) and concentrated using successively Amicon PM10 membrane and Centricon PM10 membrane. Finally, they were dialysed extensively against 100 mM sodium acetate pH 5.5. Their purity estimated by SDS-PAGE was > 95%. The reactivity with peptide CGGIRGERA of Fab 4x11 was checked in ELISA using anti-mouse Fab goat IgG conjugated to peroxidase to measure peptide-bound Fabs.

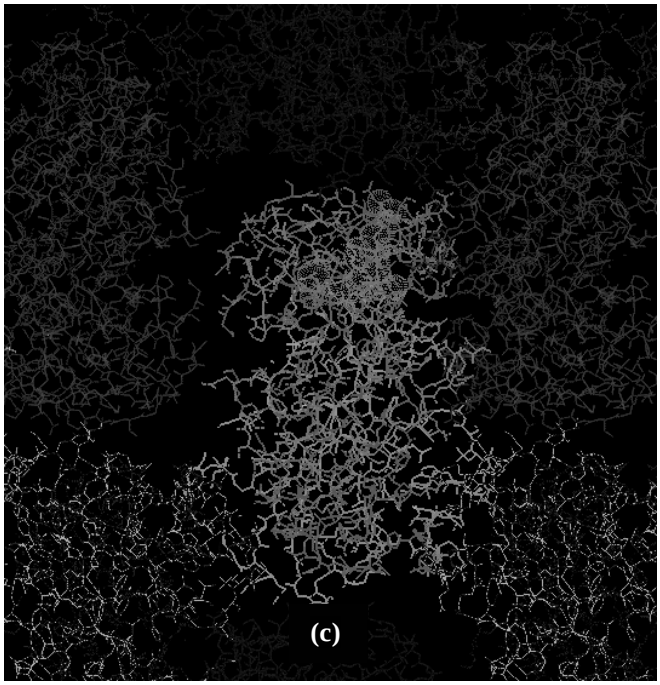
To determine the sequence of the variable regions of 4x11 antibody light and heavy chains, total RNA was isolated from hybridoma lysate using the "TRIzol reagent" (Life technologies Inc, USA). Amplification and direct sequencing of V_H and V_L hybridoma cDNA were performed following exactly the method described by Jovelin *et al.* (1995).

2.2. Crystallisation and screening

The free Fab was screened alone and together with a single site binding mutant of protein L (PpL-D55A) (Stura *et al.*, 2002a), to take advantage of the crystallisation strategy of immunoglobulin binding protein complexation (Stura *et al.*, 2001) and to verify if, at the high protein concentrations used to grow crystals, protein L has







some residual affinity from the κ2 light chains. The screening made use of six selected precipitant solutions:

(i) solution 2C; 20% polyethylene glycol (PEG) 4000, 200 mM imidazole malate, pH 7.0, from a standard screen (Stura *et al.*, 1992). The results obtained from this initial test after 5-10 min is used to determine the concentration of the subsequent precipitant solutions screened on the first day (Stura, 1999a).

(ii) 2.0 M ammonium sulphate, 150 mM sodium citrate, pH 5.5 (condition 2D from the same screen);

(iii) 17% monomethyl PEG (MPEG) 5000, 160 mM imidazole malate, pH 6.0;

(iv) 11.5% PEG 3350, 2.5 mM zinc acetate, 1.2 mM calcium chloride, 60 mM cacodylate, pH 6.5; (v) 20% PEG 3350, 15% isopropanol, 100 mM sodium citrate, pH 5.5 and

(vi) 16% PEG 3350, 500 mM sodium chloride, 50 mM sodium borate, pH 8.5. This last condition has been very successful in the crystallisation of several Fab-PpL complexes with or without antigen (Stura *et al.*, 2002a). The final optimised crystallisation condition for the Fab 4x11-peptide complex, 16% MPEG 2000, 50 mM sodium borate, pH 9.0, is strongly related to it. The screening was done in parallel as described by Stura (2001). Crystals for data collection were enlarged by using streak seeding followed by macroseeding (Stura, 1999b).

2.3. Data collection

The crystals were vitrified in liquid ethane and stored in solid ethane in a liquid nitrogen storage tank until defrosted in the cryostream at the data collection facility. The cryosolution was 14% (w/w) MPEG 5000, 27% ethylene glycol, 1.7 mM zinc acetate, 1.7 mM $CdCl_2$, 55 mM sodium cacodylate, pH 6.5. This same solution reported here, has also been reported for other Fab crystals and their complexes (Stura *et al.*, 2002a,b). This cryosolution is well tolerated by many protein crystals. Data were recorded at ESRF beamline ID14-EH4 at cryotemperature on a Quantum4 CCD detector and processed by using the HKL package (Otwinowski & Minor, 1997). These crystals belong to the orthorhombic space group $P2_12_12$ with a = 64.2 Å, b = 114.0 Å, c = 140.39 Å. The data set used for structural analysis has 28887 unique reflections in the 20 to 2.7 Å resolution range with an overall R-merge of 6.8% (last shell 46.5% 2.76 - 2.7 Å), with 99.2% (last shell 97.4%) completeness and a 7-fold redundancy and an average I/σI of 12.0.

2.4. Structure determination

The structure was solved by molecular replacement by using the program *AMoRe* (Navaza, 1994). Two Fab were searched for sequentially. Several Fab with elbow angles differing by 5° from each other were used in the search (Wilson *et al.*, 1991). The best solutions started from 165° (correlation coefficient 32%) to 180° (correlation coefficient 38%) dropping suddenly to a correlation coefficient of 27% for 185°. Fab in this elbow angle range were searched with the best solution being obtained for PDB-ID = 1RMF (Jedrzejas *et al.*, 1995) with an elbow angle of 179.2°. The molecular replacement solution was then subjected to rigid body refinement and a cycle of positional, temperature factor and simulated annealing refinement using *CNS* (Brünger *et al.* 1998). The electron density maps were calculated and viewed with *Xfit*, part of the *XtalView* package of programs (McRee, 1999). A FASTA search was carried out against the Protein Data Base with the sequence of Fab 4x11. The closest matching Fab, PDB-ID = 1E4W (Hahn *et al.*, 2001) was

Figure 1 Crystal packing of the complex of Fab 4x11 with the cysteine-dimerised peptide CGGIRGERA. (a) View along the X-axis; (b) along the Y-axis and (c) along the Z-axis.

superimposed on the heavy chain using the least square fit routine of *Xfit* and the sequence modified either to the correct residue or truncated to alanine depending on the quality of the electron density for the side chain. The cyclic peptide present in the anti-TGFα Fab complex (PDB-ID: 1EAW; Hahn *et al.*, 2001) was docked into the electron density for the CGGIRGERA peptide in the 2Fo-Fc σA map. The peptide residues that fitted the density were truncated to alanine and their fit improved with *Xfit*. Later the side chains were rebuilt once the density for the side chains became evident following refinement with CNS (Brünger *et al.*, 1998).

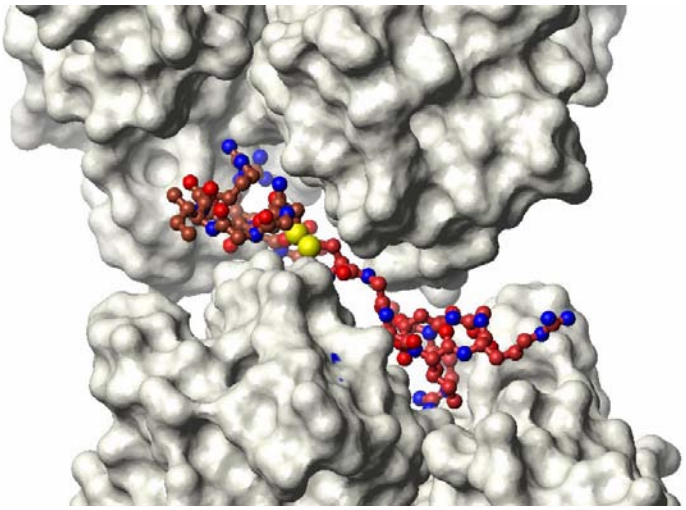


Figure 2 Surface representation of Fab 4x11 with the CGGIRGERA peptide in a ball-and-stick representation showing the importance of the disulphide bridge for bringing together the two Fab in the asymmetric unit.

3. Results and discussion

3.1. Crystallisation screening

The first crystals were obtained with solution (iv) 11.5% PEG 3350, 2.5 mM zinc acetate, 1.2 mM calcium chloride, 60 mM cacodylate, pH 6.5. These crystals grew in the presence of several peptides. The crystals were extremely thin hairline needles which could be propagated by streak seeding but could not be improved. The second crystal form was found only later by screening both with PpL-D55A and the CGGIRGERA peptide. They were first obtained from 16% PEG 3350, 500 mM sodium chloride, 50 mM sodium borate, pH 8.5. They are difficult to obtain and to grow. Although, data has been collected to 2.7 Å, the crystallisation efforts are continuing to better understand the problems associated with this crystallisation.

Given that a disulphide-dimerised peptide is present in the lattice, we are examining the hypothesis that insufficient dimeric peptide is present in solution. The crystallisation works best at pH 9.0. At this pH oxidation of the cysteine is enhanced. By adding 5-10% DMSO, it has been possible to increase the nucleation rate, suggesting that insufficient dimeric peptide is present in solution. In the presence of DMSO the rate in nucleation is increased but crystal growth is still severely inhibited. This could be due to the presence of Fab without a bound dimeric peptide. No experiments have yet been carried out with purified dimeric peptide.

To study the effect of linker length we have synthesised one shorter and one longer peptide: CGIRGERA and CGGGIRGERA. Neither has resulted in crystals under the same conditions even when seeded with co-crystals of Fab 4x11-CGGIRGERA. Similarly no crystals have been obtained under these conditions with peptides of different length containing the ^{130}IRGERA^{135} epitope (i.e. peptides 127-135, 124-135, 122-135 and 120-135) nor with peptides with the CGGIRGERA sequence where either the peptide bond between E-R or R-A was reduced. It appears that the lattice formed by Fab 4x11-CGGIRGERA is very selective for this particular peptide. In conclusion, some antibodies are hard to crystallise because they are unable to form strong lattice contacts. Fab 4x11 is among them. The final objective of the study is to understand the cross-reactivity of this antibody. It is therefore essential that crystals should also be obtained with other antigens, dimeric of otherwise.

3.2. Crystal packing

The asymmetric unit contains two antibodies related by a non-crystallographic two-fold rotational axis. The non-crystallographic symmetry is reflected in the data: at low resolution, the even h+k+l reflections are stronger than the odd (<even h+k+l> / <odd h+k+l> = 1.85). The two antibodies in the asymmetric unit are positioned so that their binding sites are face-to face. The crystal contacts made by the Fab are not extensive without considering the bound peptide. Once the peptide is also taken into consideration, it becomes obvious by visual inspection, that the contact mediated by the disulphide bridge is more extensive than any other crystal packing contact (Figure 1). The importance of the peptide and the disulphide bridge in bringing the two Fab in the asymmetric unit together is clearly shown in Figure 2.

Since PpL-D55A mutant was used in the crystallisation, we have superimposed the Fab'-PpL-D55A on Fab 4x11 and analysed the Fo-Fc electron difference maps. Consistent with the facts that this Fab has a κ2 light chain, and that PpL does not bind to, or binds extremely weakly to κ2 light chains there is no strong density for PpL in the Fo-Fc electron difference maps. However, PpL-D55A could be accommodated in the lattice without steric clashes and hence we cannot exclude at this stage in the refinement that there could be low occupancy PpL binding. Its presence would make the crystal packing more extensive. In the crystallisation, PpL is not needed to grow crystals although the presence of PpL in the crystallisation drop enhances nucleation. A phenomenon which could be explained by weak PpL binding. The resultant packing is very satisfying since it explains most of the observations made during the crystallisation efforts.

4. Use of disulphide dimerised protein for protein crystallisation

The fact that it is difficult to get crystals of Fab 4x11, makes it an interesting antibody to use as a model for novel screening methods. This crystallisation has confirmed that the crystallisation of peptides with cysteines is extremely problematic. It is advisable to reduce the cysteine with iodoacetamide before undertaking the crystallisation and to purify the peptide dimer and screen using both peptides. Even with seeds and refined crystallisation conditions, crystals cannot be obtained reliably. Nonetheless the study of antigenic peptides in the formed used in the coupling to the carrier protein is important to determine the contribution the linker makes to binding (Monnet *et al.*, 2002) and hence it is desirable to obtain a crystal structure of the same peptide as was used in the immunisation.

This result can be extended to other proteins, whereby exposed cysteines could be engineered on the surface of proteins, as a way of crystallising proteins which are otherwise difficult to crystallise. Some further developments will undoubtedly be needed to make the technique useful for crystallization of the dimeric protein linked by the disulphide. The Fab-peptide complex is a first step in the determination on the development of such methodology. This is likely to involve the chromatographic separation of the dimeric protein and to crystallise the monomer, iodoacetamide chemical

treatment might be advisable except when growing crystals for heavy atom derivatisation with a mercury compound.

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