Protein L mutants for the crystallization of antibody fragments

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In many cases, antibody and their complexes can be crystallized and their structure determined without major difficulties. The remaining problematic cases may be approached through techniques such as of combinatorial complex crystallization which uses immunoglobulin binding proteins (IBP). The range of lattices that can be made using this method can be expanded by engineering mutants of IBP domains. We have designed *Peptostreptococcus magnus* protein L (PpL) mutants with altered immunoglobulin light chain binding characteristics. While the wild type PpL has two binding sites, some of the mutants contact the light chain via only one site. Other mutants have combinations of weakened first and second binding sites that modify their crystallization properties and their packing mode. In this study, we have selected PpL mutants with different behavior and that are most useful for crystallization and we present the various packing modes obtained so far.

Keywords: Antibody crystallization; immunoglobulin binding protein; crystal engineering ; protein L mutants

1. Introduction

Antibody complexation has been gaining importance as a method to grow crystals of macromolecules that cannot be crystallized alone. The potential of this method can be further increased by combinatorial complex crystallization (CCC) (Stura *et al.*, 2001a) which consists in the use of immunoglobulin binding protein (IBP) domains. These IBP domains bind outside the antigen combining site of the antibody without disrupting antigen binding. Then, the addition of an IBP domain changes the exposed immunoglobulin-antigen surface and allows for different packing contacts to be formed. This makes these domains ideal for protein co-crystallization. A domain from streptococcal protein G has already been used to obtain crystals of an Fab-peptide complex (Derrick *et al.*, 1999) and we have had good success with using a domain from *Peptostreptococcus magnus* protein L (PpL) in the crystallization of a glycoprotein-Fab complex (Graille *et al.*, unpublished results). To better understand the molecular details of IBP binding to antibody heavy and light chains, we have determined the crystal structures of two complexes between a human Fab and two IBP: domain D from *Staphylococcus aureus* protein A (SpA) and a domain from PpL (Graille *et al.*, 2000, 2001). Of these two domains, PpL represents a better reagent for crystallization since it recognizes 50% of human and more than 75% of murine kappa chains, while SpA binding to Fab is restricted to VH3 heavy chains (i.e. 45% of human but only 10% for mouse antibodies). The crystal structure of the human Fab-PpL complex shows that PpL has two binding sites for light chains and preliminary centrifugation studies suggest that the affinity of the second site is about one order of magnitude lower than the first one (Graille *et al.*, 2001). The two binding sites of the wild type domain of PpL recognize the same region of immunoglobulin light chains so that they compete for light chain binding. As a result, the weaker second site would be unused except in the presence of excess Fab. In this case, a PpL domain would be sandwiched between two Fab and not contribute to the formation of lattice contacts (Figure 1). The elimination of one of the two binding sites allows PpL to contribute to crystal formation in several different ways. Then, it should be possible to select only one binding interface, either by stoichiometry variation screening (SVS) (Stura *et al.*, 2001b) or by the use of the designed mutants, that can be used to vary the contacts made by PpL in the crystal.

1.1. Strategy in mutant design

Each of the three IBP: SpG, SpA and PpL, that bind to the Fab portion of immunoglobulins, can be subjected to site directed mutagenesis to yield novel reagents with altered crystallization characteristics that can aid in the crystallization of antibodies and their complexes. This includes different dimerization modes, altered surface residues and strengthened binding. This could be extended to double domains which could be used to bring together different Fab regions to help in the formation of crystals contacts. PpL was chosen because of its wide recognition of murine and human antibodies and our work has been limited to single PpL domains. We have started by the deletion of one of the two PpL independent binding sites, to abolish PpL bivalency. Without this first step most mutations are likely to be ineffective since PpL is sandwiched between two Fabs and cannot contribute to the formation of crystal contacts. With the disruption of one of the two binding sites, most mutations of surface residues will potentially yield useful reagents for crystallization. Surface residues are a good target since their mutation is unlikely to disrupt folding and will alter the propensity of the PpL-Fab-antigen complex to crystallize. Other interesting mutants are those that are still able to dimerize but where site 1 has reduced affinity for antibodies so that the dynamic of crystal growth is altered. After testing two such mutants, we find differences in the crystallization trials of complexes between Fab and antigens.

2. Materials and methods

2.1. Preparation of Fab' 19D9D6

Monoclonal murine antibody 19D9D6 (IgG1, V_Lk9; Jolivet-Reynaud *et al.*, 1998) was cultured, sequenced, expressed, cleaved and purified as described in detail elsewhere (Graille *et al.*, submitted). Briefly, the IgG was purified on a protein A column and then cleaved with pepsin to yield $F(ab')_2$. The $F(ab')_2$ were purified by size-exclusion and reduced to Fab' fragments using β-mercaptoethanol. The free cysteines were then blocked with iodoacetamide. The Fab' was further purified by size-exclusion chromatography.

2.2. Mutagenesis and purification of the PpL mutants

All the mutants are made starting from the Y64W template DNA (Beckingham *et al.*, 2001). The Y64W mutation is used to analyze the properties of the mutants in solution by stopped flow fluorescence. The mutants discussed here are D55A, Y53F, L57H and D55A/L57H. These mutants were expressed in *Escherichia coli* as previously described (Beckingham *et al.*, 2001) and stored in saturated ammonium sulfate at 30 mg/ml at 4ºC. PpL Y53F and L57H are site 1 mutants, D55A is a site 2 mutant and D55A/L57H has a mutation at both sites 1 and 2 (for a detailed biochemical discussion see Graille *et al.*, 2001).

Table 1 Data collection from crystals of Fab' 19D9D6 with PpL mutants.

Data Set	D55A	L57H	Y53F	D55A/L57H
Crystallization	*90% WS3*	*52% WS1*	*50% WS1*	*90% WS3*
drops (protein / WS)	2.4 / 1.8	2.0 / 2.0	2.0 / 2.0	2.0 / 2.0
cross-seeding	yes (free Fab)	no	yes (L57H)	yes (L55A)
Data Collection	ESRF-ID14EH1	ESRF-BM30A	ESRF-ID14EH1	ESRF-ID14EH1
Space group	P212121	P21	P21	P21
unit cell: a, b, c (Å)	78.5, 101.0, 149.1	73.6, 95.5, 95.8	73.2, 94.8, 94.8	43.6, 230.5, 123.6
α, β, γ (°)	90.0, 90.0, 90.0	90.0, 106.5, 90.0	90.0, 106.5, 90.0	90.0, 91.7, 90.0
resolution range(Å)	20.0 - 2.1	20.0 - 2.4	20.0 - 3.1	20.0 - 3.8
completeness (%)	97.6	98	99.5	99.5
multiplicity	6.77	3.15	3	3.5
<I/σI>	26.6	11.7	4.2	2.0‡
Rsym (%)	6.7	9.8	17.3†	17.3‡
Refinement	*CNS*	*CNS*	*CNS**	*CNS**
R-free (%)	24.7	24	29.3*	34.9*
R-work (%)	19.7	19	23.1*	25.9*

* Refinement in progress using CNS (Brünger *et al.*, 1998).
† Crystal quality is affected by a certain degree of polycrystallinity.
‡ Very weak data.

2.3. Crystallization

Complexes were prepared in solution before the crystallization trials, by mixing 18 µl of Fab' 19D9D6 (6 mg/ml) with 4 µl of PpL mutant (16 mg/ml). PpL stored at 32 mg/ml as an ammonium sulfate precipitate was diluted ten fold and re-concentrated 3 times in 0.05% sodium azide to reduce the salt present. Screening for crystallization was carried out in sitting drop vapor diffusion using Q-plates with multiple drops per reservoir (Stura, 2001) so that various mutants could be tested in parallel over the same precipitant reservoir. Crystals were grown by vapor diffusion at 17°C in an air conditioned room, in sitting drops. Three working solutions based on previous conditions used in the crystallization of other Fab were used in the screening and to grow crystals: *WS1* (24% w/w monomethyl polyethylene glycol (MPEG 5000), 500mM sodium chloride, 200 mM Tris-HCl, pH 9.0), *WS2* (14% w/w MPEG 2000, 10% ethanol, 200 mM imidazole malate, pH 7.0) and *WS3* (10% w/w MPEG 5000, 100 mM sodium acetate, pH 4.5). Solution *WS1* is related to that used in the crystallization of Fab 2A2 in complex with wild type PpL (13% - 16% MPEG 5000, 100 mM imidazole malate, pH 8.5; Graille *et al.*, 2001) and to the solution used for Fab 4x11 (16% w/w MPEG 2000, 50 mM sodium borate, pH 9.0; Stura *et al.*, 2002a). The initial crystallization screening was carried out with the Fab alone in a manner analogous to that used for Fab 4x11 to determine the typical amount of precipitant needed to precipitate the Fab'. Fab' 19D9D6 needs from 9% to 12% PEG so that the original *WS1* is diluted by 50%, *WS2* is diluted by the same amount as PEG and ethanol act as co-precipitants. *WS3* was designed specifically for PpL mutant D55A in complex with this Fab' and is used as is or as a 90% dilution. The size of the protein drops ranged from 1.2 to 3.6 µl, which were placed on the sitting drop coverglass of the Q-plate (Stura, 2001) in advance of the precipitant, which was then layered on the protein drop without mixing. Typically precipitant drops ranged from 1.2 to 2.4 µl. Often unequal drop sizes of protein and precipitant were used in order to start and finish with different protein and precipitant concentrations in the drop. Although, Fab' 19D9D6 crystallizes relatively easily, yielding a large variety of lattices under very similar conditions, in many cases the crystals obtained diffract poorly, have high mosaicity and tend to be polycrystalline. Typically, the data collected from the first crystals obtained for any particular complex is of poor quality. After one or two rounds of crystal improvement by streak seeding followed by macroseeding (Stura, 1999) better data can be collected. For example, the first crystals obtained for the PpL D55A mutant complex were polycrystalline and unusable for data collection, the second attempt yielded data to 2.6Å, later crystals extended the resolution to 2.1 Å. The statistics shown for Fab' 19D9D6 in complex with PpL mutants Y53F and D55A/L57H (Table 1) exemplify the tendency observed for this Fab' whether complexed or not.

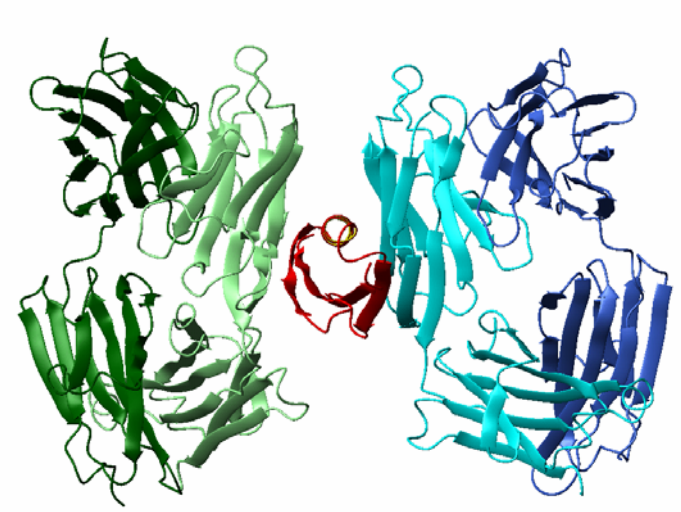

Figure 1 Schematic representation of the two-site complex formed between PpL mutants and Fab' 19D9D6. Packing of PpL between two Fab, typical of two-site binding. This packing mode is found for wild type PpL and for mutantsY53F and L57H from which the figure has been obtained.
Data collection and structure determination

For all Fab' 19D9D6-PpL mutant complexes presented here, data were collected from crystals transferred, as described in (Stura & Gleichmann, 1999), into a cryo-protectant solution containing 5 µl ethylene glycol, 3 µl 50% w/w MPEG 5000 and 10 µl (10% w/w MPEG 5000, 3 mM $ZnCl_2$, 3 mM $CdCl_2$, 100 mM sodium cacodylate pH6.5). All data were processed using the *HKL* package (Otwinowski,1997).

For Fab' 19D9D6 - PpL-D55A complex crystals, data were first recorded at 100K to 2.6Å on beamline ID14-EH1 and later, after improvements in crystal quality, to 2.1Å resolution on ID14-EH4 at the ESRF synchrotron facility (Grenoble, France). These crystals belong to the orthorhombic space group $P2_12_12_1$ (Table 1). The initial 25-2.6 Å data set was used to solve the structure of the D55A mutant by the molecular replacement method with the program *AMoRe* (Navaza, 1994) using the coordinates from the variable (VL/VH) and constant (CL/CH1) regions of the uncomplexed Fab' 19D9D6 structure solved at 3.4 Å resolution from crystals belonging to the space group $P2_1$ with cell parameters a = 75.8 Å; b = 91.6 Å; c = 81.3 Å; β = 113.5°. This structure was solved again using the

program *AMoRe* (Navaza, 1994) from data at 3.9 Å collected from crystals belonging to the space group $P2_12_12$ a = 60.8 Å; b = 170.8 Å; c = 40.8 Å. In advance of the availability of the light and heavy chain sequences, the model used, 1UCB (Sheriff *et al.*, 1996), was selected because of the similarity in space group and cell parameters: $P2_12_12$; a = 61.1 Å; b = 174.3 Å; c = 45.8 Å. With the availability of the sequence, a FASTA search was carried out against the PDB data base and the heavy and light chains replaced by those from 1DBA (Arevalo *et al.*, 1993) and 1HIL (Rini *et al.*, 1992) respectively. The sequence was then modified to conform to that determined for Fab' 19D9D6. The two uncomplexed Fab' 19D9D6 data sets were collected on the home source with capillary-mounted crystals. Through seeding to obtain better crystals, with cryogenic vitrification in liquid ethane, and synchrotron data collection on beamline ID14-EH1 at the ESRF synchrotron facility the data for the uncomplexed Fab' 19D9D6 was improved to a final 1.6 Å resolution ($P2_12_12$; a = 63.5 Å; b = 173.7 Å; c = 41.7 Å). The molecular replacement located correctly the two Fab' in the asymmetric unit, but attempts to position D55A-PpL by molecular replacement were unsuccessful. Hence, after an initial refinement of this model, PpL was positioned into σA-weighted 2Fo-Fc and Fo-Fc electron density maps. The programs *XtalView* (McRee, 1999) and *TURBO* (Roussel & Cambillau, 1989) were used for electron density map interpretation and other graphical analysis. Further details of the structure determination and refinement of Fab' 19D9D6 complex with PpL mutant D55A are given in (Graille, *et al.* submitted).

Data for the Fab' 19D9D6-L57H PpL complex were collected to 2.4 Å at beamline BM30A at the ESRF synchrotron facility (Table 1). Two Fab' 19D9D6 can be positioned by molecular replacement as described above, but not PpL-L57H. After refinement of the two Fab', the Fo-Fc electron density map contoured at 3σ revealed the presence of one PpL domain sandwiched between the V_L regions of the 2 Fab' in the asymmetric unit, in a manner similar to the human Fab 2A2-PpL wild-type structure (Graille *et al.*, 2001) (Figure 1).

Data for the Fab' 19D9D6-Y53F PpL complex were collected for the first time on beamline ID14-EH1 at the ESRF synchrotron facility to 3.1Å resolution (Table 1). The cell parameters obtained are very similar to those for the Fab' 19D9D6-L57H PpL complex and so is the packing of the Fab'-PpL mutant complex. This allowed the structure to be solved by rigid body refinement from the coordinates of the Fab' 19D9D6-L57H PpL complex. As was needed for the D55A PpL mutant Fab' complex, rounds of crystal improvement will be carried out for this complex in order to obtain better diffracting crystals.

Crystals of Fab' 19D9D6-D55A/L57H PpL complex grown in the presence of a 45 residue peptide were used for preliminary data collection on beamline ID14-EH1 at the ESRF synchrotron facility to determine whether the peptide was bound at the active site before undergoing the standard series of crystal improvements by streak- and macro-seeding. The data is usable only to 3.8 Å (Table 1). The structure was solved by molecular replacement using the Fab' 19D9D6-D55A PpL as the search model using *AMoRe*. Three Fab'-PpL complexes are found by the program. Two of the Fab complexes form a dimer with two PpL molecules sandwiched between them (Figure 3). The fourth Fab'-PpL complex can be built by least square superposition of the dimeric complex (Fab'-PpL-PpL-Fab'; Figure 3) onto the unpaired Fab PpL complex. The so constructed model was subjected to rigid body refinement using *CNS* (Brünger *et al.*, 1998). The R-free of the model constructed in this manner descended to 38.5% while these values remained 8% higher without the fourth Fab'-PpL complex. The current R-free and R-work, (in the absence of antigen) are 34.9% and 25.9% respectively (Table 1), while these values are higher (39.8% and 29.8% respectively) without PpL and crystal packing would be defective. Again, these crystals need the usual cycles of seeding to improve the resolution and quality of the data.

Figure 2 Single site binding of mutant D55A. Only site 1 is used, the remaining surface of PpL is available for crystal contacts and can be mutated to increase the number of lattices that may be obtained.

3. Results and discussion

3.1. Crystal packing

Both complexes with PpL mutants L57H and Y53F with Fab' 19D9D6 show two binding sites for PpL (Figure 1). The binding mode is indistinguishable from that observed with the wild-type PpL and Fab 2A2 (Graille *et al.*, 2001) confirming that the Y64W mutation does not disrupt binding at either site 1 or 2. The importance of Asp 55 is evident since both the single D55A mutant (Figure 2) and the double mutant D55A/L57H (Figure 3) result in suppression of the second binding site. In the case of D55A/L57H, the PpL mutant dimerizes and forces the dimerization of the Fab' to which it is bound via PpL site 1. The overall packing has four Fab'19D9D6-D55A/L57H PpL complexes in the asymmetric unit arranged as two PpL-Fab' 19D9D6 dimers (Figure 3b). Of the four potential antigen binding sites, two are facing each other and their proximity could result in a certain degree of interference. The two other antigen binding sites are unhindered and can bind antigen.

3.2. Crystallization screening

The fact that the binding of each mutant affects the crystallization characteristic of the antibody-antigen complex in a different manner, is particularly useful to tailor the crystallization properties simply by using distinct PpL mutants. For example, PpL mutants Y53F and L57H, both bind with both sites to the Fab' giving rise to the same

packing in the absence of antigen, but when antigen is added they do not produce the same crystals. This could be explained on the basis that the Y53F mutation weakens site 1 binding more than the L57H mutation, but how this affects crystal growth is not immediately obvious. However, the difference in behavior is welcome as it increases the chances of crystallizing complexes even if it may be necessary to screen each mutant through the complete combinatorial screen to arrive at crystals. Thus, to reduce the complexity of the combinatorial screening it is important to continue using a limited number of precipitants solutions used (*WS1* at basic pH, *WS2* at neutral pH and *WS3* at acidic pH). This has worked well so far with this Fab' and for other Fab with two-site wild type PpL. All the optimized crystallization solutions for PpL double site binding are closely related to *WS1*.

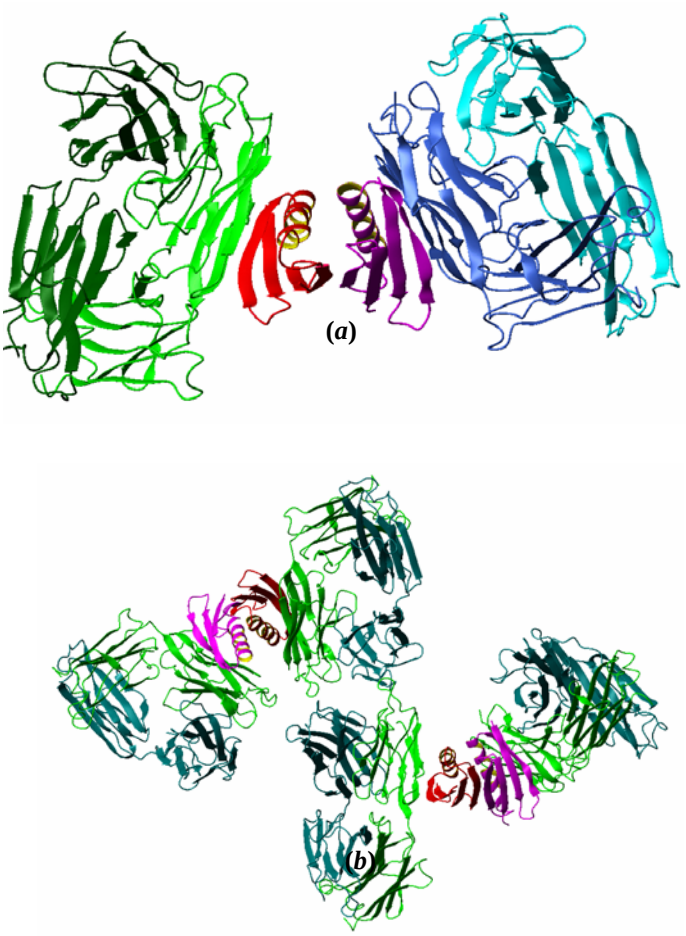

Figure 3 PpL Dimerization. (a) Dimerization of Fab' 19D9D6 mediated by contacts between two PpL molecules of mutant D55A/L57H. (b) Four Fab' are present in the lattice, two antigen binding sites can bind antigen unhindered.

3.3. Variation of stoichiometry

Because PpL is small compared to the Fab, to improve the accuracy in stoichiometry, the Fab-antigen complex is first mixed with PpL in a 1:1 ratio (in larger volumes) and then combined with Fab-antigen complex (without PpL) to achieve ratios of 1 PpL : 2 Fab, while setting up the drops. So far we have not found any lattices with 1 PpL bound to two Fab and an extra Fab without PpL (a 1 PpL : 3 Fab stoichiometry), equivalent to the lattice found with SpA (Graille, *et al.*, 2000). Such stoichiometric ratios are possible. Another likely non-stoichiometric lattice could be built with a PpL dimer (mutant D55A/L57H; Figure 3) sandwiched between two Fab and an Fab without PpL giving a stoichiometry of 2 PpL and 3 Fab. Stoichiometry variation screening will continue to be an integral part of the screening for complexes (Stura *et al.*, 2001*b*). We have tried to use the wild type PpL for single site binding using PpL wild type in excess. Because site 1 and site 2 compete for essentially the same binding site on the immunoglobulin light chain, when excess PpL is used, binding should occur only via PpL site 1, since this site is stronger than site 2. However, this approach has not given good results. The crystals that have been obtained in this manner tend to be polycrystalline, while single crystals can be obtained with the mutants with the deleted site 2.

4. Conclusions

In conclusion, PpL mutants facilitate Fab-antigen crystallization in two distinct ways: (i) by bringing together two Fab that then establish crystal contacts, and (ii) by making crystal contacts with other components of the asymmetric unit, including itself. All mutants tested so far have yielded diffracting crystals of the Fab-PpL complex without antigen and in two cases PpL has helped crystallization of the Fab-antigen complex. More mutants will be tested in the future for their ability to create a greater variety of lattices, both with antigens of largely varying sizes and without antigen. Mutants with a free cysteine have also been made, to be used for heavy metal derivatives or sulfide-bridge mediated dimerization (Stura, *et al.*, 2002a). The PpL mutants are also important for developing combinatorial based scaffold systems for protein crystallization (Stura, *et al.*, 2002b). The various crystal packing that can be obtained will be very important in this effort.

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