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# Crystallization and preliminary X-ray diffraction studies of mutants of B1 IgG-binding domain of protein L from *Peptostreptococcus magnus*

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The small 62-residue IgG-binding domain B1 of protein L from *Peptostreptococcus magnus* (Ppl-B1) has proven to be a simple system for the study of the thermodynamics and kinetics of protein folding. X-ray diffraction studies have been initiated in order to determine how the thermostability, folding and unfolding rates of a series of point mutations spanning Ppl-B1 correlate with the high-resolution structures. To this end, a tryptophan-containing variant of Ppl-B1 (herein known as wild type) and two mutants, Lys61Ala and Val49Ala, have been crystallized. Full data sets have been collected for the wild type and the Lys61Ala and Val49Ala mutants to resolutions of 1.7, 2.3 and 1.8 Å, respectively. Interestingly, all three crystallize using different precipitants and in different space groups. This may be a consequence of the relatively large effects of single-site mutations on surface-charge distribution or structural conformation, which might affect crystal contact sites.



## 1. Introduction

The effect of a single amino-acid substitution on protein stability depends highly on the location of the mutation site and its environment in the protein structure. Often unpredictable interactions such as the formation of new hydrogen bonds, hydrophobic interactions or effects on overall fold would not be evident without high-resolution structures (Matthews, 1995, 1996; Ohmura *et al.*, 1997; Xu *et al.*, 1998; Yamagata *et al.*, 1998). A simple model system is needed to gain predictive parameters relating structure to amino-acid substitutions. We have initiated studies to correlate high-resolution protein structures with available thermodynamic and kinetic data for a small globular protein, the B1 domain of protein L (Ppl-B1).

Protein L is a multidomain cell-wall protein in *P. magnus*. The B1 domain of protein L is a small 62-residue immunoglobulin G (IgG) binding domain (Kastern *et al.*, 1990) which lacks proline residues or disulfide bridges. The NMR structure of the B1 domain of protein L revealed a $\beta\beta\alpha\beta\beta$ fold (Wikström *et al.*, 1994). The crystallization of an 84% homologous C3 domain of protein L from a different strain of *P. magnus* has also been reported (Sohi *et al.*, 1995); however, the structure has not yet been published.

The folding and unfolding of the B1 domain of protein L has been extensively studied by various methods (Gu *et al.*, 1997, 1995; Scalley *et al.*, 1997; Yi & Baker, 1996). Recently, a systematic analysis describing the stability ($\Delta G$) and folding kinetics for 70 point mutations using a Tyr47Trp variant of Ppl-B1 have been described (Kim *et al.*, 2000). To serve as a benchmark for the analysis of mutant structures, we have initiated the structure determination of the Tyr47Trp-containing pseudo wild type by crystallization and the collection of high-resolution diffraction data. In addition, we report the crystallization of and data collection from two mutant proteins, Val49Ala and Lys61Ala. Lys61Ala showed thermostability and folding kinetics similar to the wild type, whereas Val49Ala unfolds 2.5 times faster and has a $\Delta\Delta G$ of 2 kJ mol$^{-1}$ compared with the wild type (Kim *et al.*, 2000).

## 2. Results

### 2.1. Cloning and purification

For simplicity, residues 94–155 of Ppl-B1 are renumbered 1–62. The mutagenesis for the Tyr47Trp wild type and all subsequent mutations as well as general expression and purification procedures have been described previously (Gu *et al.*, 1995). The construction of Lys61Ala and Val49Ala are described in Kim *et al.* (2000). In short, the histidine-tagged Ppl-B1 coding region was cloned into a modified pET-15b (Novagen) vector. The protein was expressed in the BL21(DE3)pLysS (Novagen) cell line induced with 1 m*M* IPTG at mid-log growth. Purification to near homogeneity, as measured by SDS–PAGE, was achieved with $Zn^{2+}$-bound Pharmacia chelating resin. Typically, 30 mg of pure 8 kDa

**Table 1**
Unit-cell parameters and data-collection statistics for the wild type and the Lys61Ala and Val49Ala mutants.

| Data set | Wild type | Lys61Ala | Val49Ala |
|---|---|---|---|
| Space group | $P2_12_12_1$ | $P622$ | $P4_1$ or $P4_3$ |
| Unit-cell dimensions (Å) | $a$ = 51.57, $b$ = 54.02, $c$ = 95.66 | $a = b$ = 92.01, $c$ = 70.59 | $a = b$ = 53.134, $c$ = 115.514 |
| Resolution (Å) | 1.7 | 2.3 | 1.8 |
| Number of unique reflections | 28841 | 8278 | 29286 |
| $\langle I/\sigma(I)\rangle$ | 16.7 | 42.1 | 30.6 |
| $R_{\rm merge}$ | 0.074 | 0.062 | 0.047 |
| Completeness (%) | 96.2 | 99.9 | 98.7 |

protein, as examined by SDS–PAGE, was obtained from 1 l of cell culture. However, when the protein was examined using isoelectric focusing (IEF) gels, two distinct bands emerged which were 0.4 isoelectric point (pI) units apart (*e.g.* wild type pI = 6.0, 5.6). The predicted pI for the His-tagged Ppl-B1 is 6.0; however, one possible explanation for the two Ppl-B1 IEF bands is that a spontaneous $\alpha$-*N*-6 phosphogluconoylation of the His-tag in *Escherichia coli* might have produced the modified protein with the pI of 5.6 (Geoghegan *et al.*, 1999).

Ppl-B1 containing the modified His-tag was then separated by a cation-exchange step by first dialyzing the protein overnight into 100 m*M* citrate pH 5.0, 100 m*M* NaCl, 2 m*M* EDTA. The protein was loaded onto an UNO-S12 (Biorad) cation-exchange column and was eluted with an increasing NaCl gradient. The eluted protein was dialyzed overnight into 100 m*M* NaCl, 2 m*M* EDTA. The protein could typically be concentrated beyond 50 mg ml$^{-1}$ using a Millipore centrifugal filter (molecular-weight cutoff 5 kDa).

### 2.2. Crystallization

**2.2.1. Wild type**. Wild-type crystals of Ppl-B1 were grown using the hanging-drop vapor-diffusion technique by mixing equal volumes of protein (40 mg ml$^{-1}$) with precipitant [29% PEG 8000, 0.18 *M* $(NH_4)_2SO_4$ pH 6.5] at 277 K. Crystals appeared after three weeks, with typical dimensions of 0.3 × 0.3 × 0.2 mm. Transfer to cryoprotectant was achieved by eight stepwise transfers of crystals to a mother liquor that contained PEG 400 [final concentration of 26% PEG 8000, 20% PEG 400, 1.5 *M* $(NH_4)_2SO_4$ pH 6.5] at 277 K. The crystals were flash-frozen in liquid nitrogen and transferred to an air stream at 100 K.

**2.2.2. Lys61Ala**. Hexagonal rod-shaped crystals of the Lys61Ala mutant of Ppl could be grown to dimensions of 0.6 × 0.3 × 0.3 mm using the hanging-drop vapor-diffusion technique by mixing equal volumes of the precipitant (1.3 *M* citrate pH 6.5) with the protein solution (5 mg ml$^{-1}$). Crystals typically appeared after one week at room temperature. The crystal was incrementally transferred into a cryoprotectant of 20% glycerol and then flash-frozen by transferring the crystals into a stream of air at 100 K.

**2.2.3. Val49Ala**. Crystals of tetragonal bipyramidal morphology were obtained by mixing equal volumes of protein (25 mg ml$^{-1}$) and precipitant [14–25% PEG 3350, 0.2 *M* $(NH_4)_2SO_4$, 100 m*M* citrate pH 4.5] in vapor-diffusion experiments and incubating at room temperature. Crystals typically appeared after 3 d, but the largest crystals appeared from lower PEG concentrations after two weeks. The largest crystal had dimensions of 0.3 × 0.4 × 0.7 mm, but most crystals had dimensions of 0.3 × 0.3 × 0.4 mm or smaller. The cryo-preservation technique, flash-freezing of the crystal and data collection were performed as for the Lys61Ala mutant.

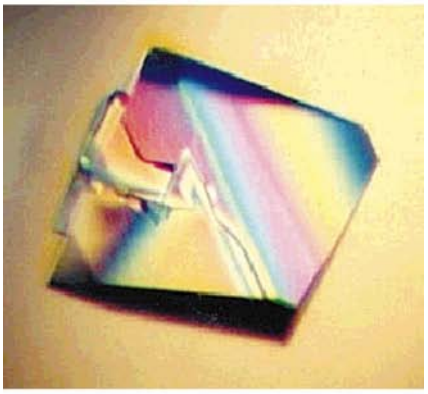
### 2.3. Data collection and processing

For all the crystals, diffraction data were collected with 1° oscillation with a Rigaku R-AXIS IV image plate, using Cu $K\alpha$ radiation from a Rigaku rotating-anode generator operating at 50 kV and 100 mA (5 kW). The data were integrated and reduced with the *HKL* suite of programs (Otwinowski & Minor, 1997). Crystallographic parameters and data-collection statistics, as processed using *DENZO* and *SCALEPACK*, of the wild type and the Lys61Ala and Val49Ala mutants are summarized in Table 1.

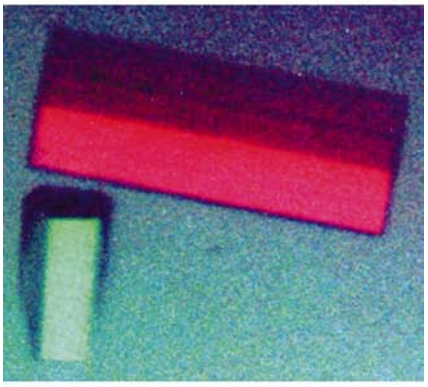
**2.3.1. Wild type**. A data set 96.2% complete to 1.7 Å with 143 903 independent observations and 28 841 unique reflections was collected. The space group is $P2_12_12_1$, with unit-cell parameters $a$ = 51.57, $b$ = 54.02, $c$ = 95.66 Å, $\alpha = \beta = \gamma = 90°$. There could be three or four monomers in the asymmetric unit; three monomers would give a Matthews coefficient (Matthews, 1968) of 2.78 Å$^3$ Da$^{-1}$ and four monomers would give a value of 2.09 Å$^3$ Da$^{-1}$.

**2.3.2. Lys61Ala**. A data set 99.9% complete to 2.3 Å consisting of 112 647 observations and 8278 unique reflections was collected. The crystal morphology (Fig. 1*b*) suggests a hexagonal space group and could be processed as *P*622, with unit-cell parameters $a = b$ = 92.01, $c$ = 70.59 Å, $\alpha = \beta = 90$, $\gamma = 120°$. These crystals showed a noticeable amount of anisotropic diffraction, perhaps because of the rod-shaped nature of the crystal. Assuming two monomers in the asymmetric unit, the Matthews coefficient would be 2.69 Å$^3$ Da$^{-1}$.

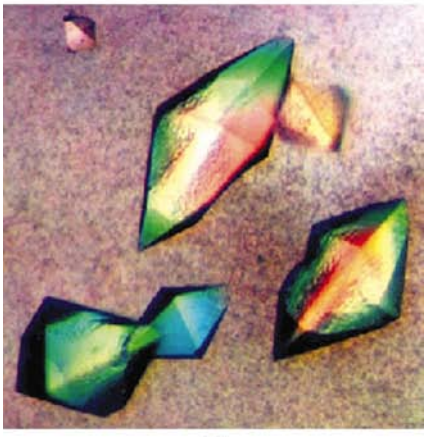
Examination of the 00*l* axis did not reveal any systematic absences and this would suggest the space group *P*622. However, the *P*622 space group is the rarest of the high-symmetry hexagonal space groups and there are only six entries in the Protein Data Bank with this space group (1bm1, 1ciq, 1coa, 1cq4, 1qhl, 2ci2). Four of the proteins that



(*a*)



(*b*)



(*c*)

**Figure 1**
Crystals of (*a*) wild type, (*b*) Lys61Ala and (*c*) Val49Ala, with typical dimensions 0.3 × 0.3 × 0.2, 0.6 × 0.3 × 0.3 and 0.3 × 0.3 × 0.4 mm, respectively.

crystallize in this space group are chymotrypsin inhibitor and its mutants, so a highly redundant data set was taken to confirm the space-group assignment *P*622.

**2.3.3. Val49Ala**. A data set 98.7% complete to 1.8 Å consisting of 134 022 observations and 29 286 unique reflections was collected in a tetragonal space group, with unit-cell parameters $a = b = 53.134$, $c = 115.514$ Å, $\alpha = \beta = \gamma = 90^\circ$. The $P4_1/P4_3$ screw axis was very clear. Consideration of the solvent content suggests that the crystal could have three, four or five monomers in the asymmetric unit. However, it is most reasonable to assume that there are four monomers in the asymmetric unit, as that would give the Matthews coefficient 2.56 $Å^3$ $Da^{-1}$.

## 3. Discussion

Single-site mutations on proteins often have little effect on crystallization conditions, as is the case with lysozyme structures (Matthews, 1995, 1996; Ohmura *et al.*, 1997; Xu *et al.*, 1998; Yamagata *et al.*, 1998). However, the small size of Ppl-B1 may heighten the effects of single-site mutations on crystallization. We were able to crystallize the wild type and two mutants, each under different conditions. It is possible that variations in the thermostability and the isoelectric point of the wild type and mutants may play a role. The wild type and Val49Ala have the same pI of 6.1 (as measured by IEF), but the $\Delta\Delta G$ for Val49Ala is $-1.2$ kJ $mol^{-1}$; however, Lys61Ala is very similar to the wild type, with $\Delta\Delta G = -0.32$ kJ $mol^{-1}$, although its pI is 5.6 owing to the loss of the basic lysine. The Val49Ala mutation is in the hydrophobic core, while the Lys61Ala mutation is on the surface of the Ppl-B1 structure (Wikström *et al.*, 1994). The core mutation Val49Ala could cause sufficient disturbance to the local interactions in the hydrophobic core and lead to significant conformational changes in the structure. The conformational change in the mutant could in turn affect the crystal packing and crystallization conditions. The surface mutation of Lys61Ala changed the surface-charge distribution significantly and could affect the crystal packing compared with the wild-type Ppl-B1. In addition, the crystallization conditions for an 84% homologous C3 domain of Ppl-B1 (Sohi *et al.*, 1995) were tried unsuccessfully.

The Ppl-B1 sequence used in our study lacks the first 16 amino acids that were disordered as seen in the NMR structure (Wikström *et al.*, 1994). The wild type and mutants of Ppl-B1 all contain the Tyr47Trp mutation that confers fluorescence which was used in the folding studies (Gu *et al.*, 1995). The crystallization of the wild-type variant is critical, as the bulkiness of tryptophan will have a cascading effect on the surrounding core-residue side chains. This makes the Ppl-B1 NMR structure (Wikström *et al.*, 1994) a poor substitute when analyzing single-site mutations. Moreover, the r.m.s.d. for the backbone atoms in the regular secondary-structure elements is 0.54 Å among the 20 alternative structures published (Wikström *et al.*, 1994), which makes it difficult to analyze small changes in the mutant structures that might affect hydrogen bonds or salt bridges. Attempts to solve the crystal structures of Ppl-B1 wild type and mutants by molecular replacement using the NMR structure of Ppl-B1 (Wikström *et al.*, 1994) have not been successful.

The high-resolution crystal structures of Ppl-B1 and its mutants will offer us an opportunity to study the interdependence of interactions such as hydrogen bonds and salt bridges on residue environment in the structure. A key element in the detailed analysis of Ppl-B1 by Kim *et al.* (2000) is the focus on the folding and unfolding rates of the 72 single-site mutants. We hope the detailed analysis of the wild-type and mutant structures and the thermodynamic and kinetic data may facilitate our understanding of the folding process.

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