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Structural alterations within native amyloidogenic immunoglobulin light chains

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Summary

Amyloid diseases are characterized by the misfolding of a precursor protein that leads to amyloid fibril formation. Despite the fact that there are different precursors, some commonalities in the misfolding mechanism are thought to exist. In light chain amyloidosis (AL), the immunoglobulin light chain (LC) forms amyloid fibrils that deposit in the extracellular space of vital organs. AL proteins are thermodynamically destabilized compared to non-amyloidogenic proteins and some studies have linked this instability to increased fibril formation rates. Here we present the crystal structures of two highly homologous AL proteins, AL-12 and AL-103. This structural study shows that these proteins retain the canonical germline dimer interface. We highlight important structural alterations in two loops flanking the dimer interface and correlate these results with the somatic mutations present in AL-12 and AL-103. We suggest that these alterations are informative structural features that are likely contributing to protein instability that leads to conformational changes involved in the initial events of amyloid formation.

Keywords

Immunoglobulin light chain; amyloid; light chain amyloidosis; X-ray crystallography; protein misfolding

Introduction

A hallmark of amyloid disease is the formation of amyloid fibrils. Although the high resolution structural details of these aggregates are not well understood, it is known that amyloid fibrils have a highly organized cross- β structure that can be generated from numerous misfolded precursor proteins.^{1,2} Light chain amyloidosis (AL) is one such amyloid disease, where the precursor proteins are monoclonal immunoglobulin light chains (LCs).³ Normally two mature immunoglobulin LCs complex with two heavy chains (HCs) to form a heterotetramer, which is then secreted into circulation as an assembled immunoglobulin molecule. Free LCs can also be secreted as homodimers, often referred to as Bence Jones proteins. In AL, monoclonal free

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LCs are secreted and form amyloid fibrils that deposit in the extracellular space of vital organs; this deposition rapidly progresses leading to organ failure and death.^{3,4} Due to the presence of multiple germline donor sequences and somatic hypermutation that is required for immunoglobulin maturation, there is a wide sequence and mutational diversity in AL, with each AL patient having a unique protein sequence.^{5,6} Mutations in AL proteins are thought to have a detrimental effect on the protein stability allowing for misfolding. Several studies have linked decreased protein stability to enhanced fibril formation *in vitro*.⁷⁻¹⁰ Despite AL sequence and mutational diversity; it is possible that there are commonalities in the mechanism of fibrillogenesis and universal structural motifs in AL.

LCs have two domains: a variable and constant domain. Each of these two domains adopts the structure of a Greek key β -barrel, also called the immunoglobulin fold with 9 β -strands (A-G) arranged in an antiparallel fashion except β -strands A and G that interact in a parallel way. Within the variable domain, the β -sheets form the framework region (FR) while some of the loops form part of the complementarity determining region (CDR), responsible for antigen binding. In AL, it has been reported that the majority of the patients have amyloid fibrils formed predominantly by the variable domain.¹¹ For this reason, most reports of amyloidogenic light chains only include the variable domain (Figure 1).

Crystal structure studies of amyloidogenic light chains have previously been reported.^{10, 12-18} In our own studies using the AL protein AL-09, which differs from its germline sequence, κ I O18/O8, by seven amino acids, we have shown that large structural changes can occur. The monomeric structure of AL-09 is fairly conserved. The AL-09 dimer, however, was found to be rotated 90° from the canonical LC interface observed in the κ I O18/O8 protein. The altered interface was accompanied by decreased thermodynamic stability and faster fibril formation for AL-09, compared to the non-amyloidogenic κ I O18/O8 protein.¹³ To further this study, we carried out a systematic study of dimer interface mutants. A number of 'reciprocal' mutants, which begin with the κ I O18/O8 amino acid sequence and introduce AL-09 non-conservative dimer interface mutations, were also characterized. The mutations caused the proteins to have intermediate thermodynamic stability and amyloidogenicity values compared to of AL-09 and κ I O18/O8.¹⁰

The crystallographic studies of these reciprocal mutants highlighted a second class of subtle structural deviations. These alterations were localized to the proline-40 loop (loop between β -strands C and C').¹⁰ Deviations in C α position of approximately 3-Å were seen in this loop region, upon superposition with the κ I O18/O8 model, disrupting intra and intermolecular hydrogen bonding patterns. Of particular interest was the loss of a key hydrogen bond between the carbonyl group of K42 (loop C-C') and Y87 (β -strand F) on the other molecule in the dimer pair.¹⁰ We believe this alteration in the proline-40 loop may be relevant to the formation of the alternate dimer interface seen in AL-09. Small scale perturbations have been reported in this region of amyloidogenic immunoglobulin variable domain proteins previously. BRE is an amyloidogenic LC that was previously crystallized. The 1BRE.pdb model also shows deviations in the proline-40 loop that support our observations for the AL-09 reciprocal mutants.¹⁸ In another study, Davis et al performed a mutational analysis using the non-amyloidogenic LC protein LEN as their starting point and incorporating single mutations from the amyloidogenic protein SMA. A single P40L mutation caused this mutant protein to acquire the amyloidogenicity of the protein SMA.¹⁹ From these studies, we think that there is a clear need to understand amyloidogenic light chain structure and to assess both large and subtle effects that mutations in different locations may cause.

In this study we are comparing two AL proteins from the κ I O18/O8 germline, which have 87% sequence identity. The mutations that do occur in these proteins are in different positions relative to one another. We believe that these crystallographic models will help elucidate

structural features that may be relevant to early events in amyloid formation and ultimately, disease progression.

Results

AL-12 and AL-103 show a diversity of mutations

AL-12 and AL-103 variable domains (VL) belong to the κ I O18/O8 germline. AL-12 has eight mutations: S30T, Y32H, S65R, D70H, E81A, Q90E, N93Y and Y96Q (Figure 2) as reported previously.²⁰ Seven of these mutations are non-conservative and the mutations are located throughout the structure (CDR1, β -strand C, β -strand D, β -strand E, loop E-F, β -strand F, CDR3). 6 of the 8 mutation occur in the top of the β -barrel (where the CDRs are located). AL-103, being reported here for the first time, only has four mutations; N34I, D92H, Q100P and the insertion of a proline after residue 95 that is termed 95ProIns (Figure 2). Three of the four AL-103 mutations are clustered in the CDR3 and β -strand G, while the fourth mutation (N34I) is in the dimer interface on β -strand C. The majority of mutations in AL-12 and AL-103 differed from the previously reported mutations for AL-09, which are S30N, N34I, K42Q, N53, D70E, I83L and Y87H.^{10, 13, 21} Of these mutations, N34I, K42Q and Y87H are found in the dimer interface. N34I is common for both AL-09 and AL-103. The sequences obtained for AL-09, AL-12 and AL-103 are typical of the kappa germline ($V\kappa$) AL light chains we have observed thus far in that there is no discernible patterning to frequency, position or conservation of their respective mutations²² despite attempted classification within the field.²³ The mutations observed are located throughout the VL domain in both CDRs and FRs, highlighting the somatic origin of these mutations. It is conceded that whilst FRs can incorporate sporadic mutations at a low basal level, normally it is only the CDRs which undergo somatic hypermutation during the maturation of immunoglobulin molecules. The mutation rate at the amino acid level in immunoglobulins is estimated to be 7.0% in the first and second CDRs and 2.0% in the FR.²⁴ The FR mutation rate in AL-12 is 4.6% while AL-103 FR mutation rate is 1.8%.

AL-12 and AL-103 structure and thermodynamic stability in the context of previous studies

Both AL-12 and AL-103 were produced recombinantly. AL-12 can be purified from the periplasmic space, while AL-103 forms inclusion bodies. Using analytical size exclusion chromatography, AL-12 has been shown to be monomeric at 100 μ M with a calculated molecular weight of 12,661 Da.²⁰ AL-103 is also monomeric with an apparent molecular weight of 10,499 Da at a concentration of 144 μ M (data not shown).

In order to ensure correct refolding and recovery of global structure after purification from insoluble sources, Far-UV circular dichroism (CD) spectrum for AL-103 was obtained. The spectrum showed the standard profile for a β -sheet rich structure with two minima at 218 and 235 nm (supplementary figure 1), comparing favorably with spectra from other AL proteins reported previously^{20,21}. A thermal denaturation experiment showed that AL-103 unfolds with a T_m for AL-103 of $41.6^\circ\text{C} \pm 0.5$ (Table 1). AL-103 refolds to 60-80% of the original signal. Chemical denaturation data could not be determined for AL-103 due to a lack of two-state unfolding transition using urea or guanidinium hydrochloride (data not shown).

As reported before, thermal denaturation of AL-12 was performed and compared to previous studies to ensure reproducibility of the results. The thermal denaturation of AL-12 suggests a two-state unfolding transition with a T_m of $46.1^\circ\text{C} \pm 0.2$ (Table 1). AL-12 thermal denaturation is fully reversible. AL-12 was previously found to have a melting concentration of urea (C_m) of 2.3 M and $\Delta G_{\text{folding}}$ of -3.6 kcal/mol.²⁰

Table 1 shows the thermodynamic parameters of AL-12 and AL-103 compared to the AL protein, AL-09, and the κ I O18/O8 germline protein. κ I O18/O8 is shown as a non-amyloidogenic control. It is interesting to note here that despite a high level of non-conservative mutations in AL-12 (87.5%) compared to AL-09 (42.8%), AL-12 is more thermodynamically stable based on both chemical and thermal denaturation experiments. Although incomplete, the readily available AL-103 data would also indicate no particular concordance between the number of conservative mutations and protein stability, since AL-103 presents 100% of non-conservative mutations and its T_m is comparable to AL-09. Overall, these data suggest that mutational changes from the germline sequence destabilize the protein and the level of destabilization has more to do with the location of the mutations rather than the number of non-conservative mutations in the protein. This location-specific destabilization makes AL proteins more prone to form amyloid fibrils.^{10,13, 22}

Maintenance of normal immunoglobulin fold and canonical germline like dimer

AL-12 and AL-103 were crystallized using the hanging drop vapor diffusion method. Both structures were solved using molecular replacement (MR) with the germline protein κ I O18/O8 structure (2Q20.pdb). AL-12 was found to form P6₁22 space group crystals whilst AL-103 formed P4₁2₁2 space group crystals. Both proteins present with one polypeptide chain in the unit cell with the relevant biological assembly being a dimer. AL-12 was refined to 1.8-Å resolution, with R_{work} and R_{free} values of 19.29 and 26.56%, respectively (Table 2). The AL-103 model was refined to 1.5-Å resolution with an R_{work} of 18.95% and R_{free} of 25.14% (Table 2). Both models have the characteristic immunoglobulin fold (Figure 3, A and B), suggesting that the structure of these proteins have a large tolerance for mutations in order to maintain the canonical fold.

The molecular models for both AL-12 and AL-103 monomers, when shown with their corresponding symmetry partners, form the canonical germline like dimer interface (Figure 3C). This is in stark contrast to the crystallographic structure reported for AL-09.¹³

Previously, Protein Interfaces, Surfaces and Assemblies (PISA) analysis (http://www.ebi.ac.uk/msdsrv/prot_int/pistart.html) of κ I O18/O8 had shown that approximately 80% of the interface residues occupied equivalent positions to those residues in the structures of the light chain dimers WAT, REI, LEN, DEL and BRE.^{14,15,18,25,26} Utilizing the PISA analysis with a cutoff of buried surface area > 1 Å², we determined that AL-12 and AL-103 have 81.8% and 95.2% of the same amino acids contributing to the dimer interface compared to the κ I O18/O8 dimer. This confirms that AL-12 and AL-103 retain the same canonical dimer geometry and amino acid composition in forming the dimeric interface of the germline κ I O18/O8 protein.

The exceptions that are seen in amino acid composition are as follows. AL-12 has two fewer residues contributing to the interface than does the germline protein: D1, D50, E55, and T56. They do not form interfacial hydrogen bonds and for the most part represent very little of the total interface surface area. Loss of E55 from the AL-12 interface, although a larger area; is not considered significant. The AL-103 interface analysis also breaks down in a similar manner. T56 is left out of the AL-103 interface. This omission appears to be a difference in the modeling of the residue as guided by the electron density from the experiment and is not the result of mutation (supplementary table 1).

Crystallographic results with AL-12 and AL-103 suggest that the altered conformation of AL-09 is possibly the result of the specific combination of mutations found solely within that protein. It is also possible that all AL proteins sample the altered interface but to a lesser extent and therefore we do not see it in all crystallographic work.

Both AL-12 and AL-103 have disrupted hydrogen intra-main chain bonding networks in β -strands A, B and E. It was observed that AL-12 and AL-103 both lose hydrogen bonds between T5 and Q24. Between β -strands B and E, V19 to I75 and I21 to T72 interactions are lost in AL-12 and significantly weakened in AL-103. In AL-103, there is significant disruption to hydrogen bonding patterns between β -strands C, C' and C'', in particular residues 46 to 56. This strand-loop-strand region in κ I O18/O8 has the key backbone interactions between Y49 to N53 and D50 to S52 that are lost in the AL-103 model despite the lack of mutation in this area of the model. Further still, there are side chain specific interactions in these proteins that might be relevant to the physical properties of these proteins. In the dimer interface formed by β -strands C, C', F and G, the side chains of residues N34, Y36, Q89 and Y91 form a tight network of hydrogen bonding. In AL-103 this is clearly disrupted by the N34I somatic mutation; in AL-12 the disruptive force is less clear. In the AL-12 model residues N34, Y36, Q89 and Y91 side chains were modeled differently, they do not coalesce in the same manner, possibly due to the presence of Q90E. AL-12 and AL-103 do not have germline-like interactions in this region of the dimer interface. In both models the charge interaction is lost between the side chains of Q24 and D70. In AL-12 this interaction is lost as a direct result of the D70H mutation.

It has been well documented in barnase and RNase Sa that hydrogen bonding can contribute significantly to protein stability and consequently, protein function.^{27,28} Fluctuations in protein stability of 0.5-1.5 kcal.mol⁻¹ have been observed upon loss of hydrogen bonds in RNase Sa and Sa3 even if buried rather than solvent-exposed.²⁸ We therefore consider that disruption and loss of hydrogen bonding in AL-12 and AL-103 may impact protein stability. However, loss of hydrogen bonding alone does not completely explain the measured instability of AL-12 and AL-103, when compared to κ I O18/O8.

AL-12 and AL-103 crystal structures show clear subtle structural deviations in the CDR3 region (proline-95 loop) (Figure 4). In order to systematically determine if there were other subtle alterations in the structures of AL-12 and AL-103 throughout the protein structure, root mean squared deviation (RMSD) comparisons of the structures upon C α superposition were made between the AL-12 and AL-103 structures and those previously reported for AL-09 and the non-amyloidogenic κ I O18/O8. RMSD data, although not a stringent statistical test of the structural differences presented, gives a simple and easily interpretable comparison method of the structures obtained.

When comparing AL-12 and AL-103 to the germline κ I O18/O8 structure, it is clear that there are small deviations throughout both structures (Figure 5A and B). RMSD values rarely exceed a value of one, particularly for AL-103, throughout each model. RMSD values are seen in the proline-40 loop region of AL-12, indicating some backbone deviation in this area. Both AL-12 and AL-103 have large RMSD values in the proline-95 region when compared to both AL-09 and κ I O18/O8 as shown in Figure 3C. There is a higher frequency and larger scale of variation when comparing AL-12 and AL-103 to AL-09, than when comparing to κ I O18/O8 (Figure 5C and 5D). This indicates that the molecular models for AL-12 and AL-103 fit a κ I O18/O8 like monomer structure significantly better than the AL-09.

From the electron density data for both AL-12 and AL-103, it is clear that the proline-95 loop region is unstable, with poor supporting density for the model, despite the high resolution, completeness and redundancy for each data set (Figure 6). This is reflected by high B-factor values for atoms in this region in each model. We believe that this is caused by the loop region being dynamic and free to move. In both AL-12 and AL-103, intra-backbone interactions between the Q92 carbonyl and T97 NH group are lost when compared to κ I O18/O8. It would seem that natively, this interaction serves as a lynchpin to tether the ascending and descending arms of the proline-95 loop together. In the AL-12 and AL-103 model this does not occur. It

is hypothesized that the lack of this interaction allows the loop to be more dynamic and free to move in both the models. The mutations found in the proline-95 loop region may provide the driving force for the disruption of native interactions.

Discussion

The aim of this study was to examine the crystal structure of two AL proteins. These proteins, AL-12 and AL-103 show 87% sequence identity, however, the somatic mutations they have are in different locations throughout the protein. The crystal structure models of both AL-12 and AL-103 show the formation of the canonical, germline-like, dimer interface. This is in contrast to the conformational changes found in our previous study with AL-09. As seen previously,^{10,13,18} somatic mutations cause structural differences that could be large (AL-09) or subtle (AL-12, AL-103, this study). We feel that the subtle differences that do occur are important. Both AL-12 and AL-103 show changes in the CDR3 (proline-95 loop). The model of AL-12 shows backbone deviations in the proline-40 loop. We believe that the structural alterations in these dynamic and poorly organized regions of the protein could promote changes in the dimer interactions that may allow the protein to sample non-native conformations in amyloid formation pathways.

It is generally accepted that protein stability plays an important role in amyloid formation. It is also clear that quaternary structure (dimer interface) plays a role, as it was shown for AL-09, so perhaps some mutations affect mostly protein stability, while some may affect dimer interactions, with both quaternary and tertiary structure contributing to amyloidogenesis to different extents. This has been recently shown for Transthyretin (TTR). One of the single mutants found in TTR amyloidosis, V122I TTR exhibited a destabilized quaternary structure and a stable tertiary structure, while V30M TTR presents a stable quaternary structure but unstable tertiary structure.²⁹ For AL-09, the restorative mutant AL-09 H87Y restores the canonical dimer interface and delays amyloid formation, so H87 can be considered a residue that affects quaternary structure. In the case of AL-12, 4 out of the 8 mutations are in loops, suggesting that the effect of these mutations will be primarily in tertiary structure. From the mutations in β -strands, AL-12 Y32H, is located on the N-terminus of β -strand C, the same β -strand as residue 34, mutated to an Ile in AL-103 and AL-09. We believe that mutations in β -strand C mainly alter tertiary structure and amyloid formation. AL-12 S65R and D70H are not part of the dimer interface but could affect the overall stability of the protein. We propose that these mutations may contribute significantly to amyloid formation. Finally, Q90E is in the edge of β -strand F near CDR3 and we suggest that this residue may only affect protein stability and may not have a huge effect on amyloid formation. In the case of AL-103, I34 is located in the dimer interface and we know from unpublished observations from restorative mutation analysis that AL-103 I34N restores half of the stability of the protein, similar to the effect observed by the AL-09 I34N restorative mutant, which had an intermediate effect on amyloid formation.¹⁰ The rest of the mutations in AL-103 (D92H, Pro95Ins and Q100P) are located in the CDR3 and β -strand G. AL-103 Q100P is in the dimer interface but it is located at the β -bulge in this β -strand, so we do not think this residue is contributing much to either stability or dimer interactions.

Although no direct correlation can be drawn, current indications based on thermodynamic stability^{10,13, 20,21} and previous studies^{7,8} would indicate that AL-12 and AL-103 have an intermediate amyloidogenic potential, between that of AL-09 and κ I O18/O8. The interplay between protein concentration, dimerization, fibril formation pathways and fibrillization is unclear. It has been shown that there is to some extent an inverse correlation between protein concentration and fibril formation kinetics. Qin et al. reported that at low concentration of AL protein SMA, there was an increase in fibril formation kinetics, leading to a protective dimer hypothesis.³⁰ This inverse concentration dependence observation has however also been linked

to off-pathway aggregation.³¹ The data presented here highlight the elusive effects a small number of mutations can have on protein structure. It is clear that there is subtle interplay leading to the global instability of the variable domain, conferring its amyloidogenic properties and possibly providing a route for protein misfolding. Similar conclusions have been drawn in other proteins. A recent publication by Palaninathan et. al. described small scale structural perturbations at low pH that might allow for misfolding and increased amyloidogenic potential of transthyretin.³² These results highlight that there may be commonalities in the mechanism of fibrillogenesis and universal structural motifs not only in AL but other amyloid forming proteins.

In conclusion, our crystallographic analysis has allowed us to determine that AL-12 and AL-103 are capable of forming canonical dimer structures and that some or all of their mutations in a cooperative way disrupt the tertiary structure but have little effect on the quaternary dimeric interactions that were disrupted in AL-09.

Methods

Cloning, Expression, Extraction and Purification

Patient AL-12 was cloned and sequenced as described previously.²⁰ Patient AL-103 presented with heart, liver and tongue AL deposits. AL-103 variable domain sequence was previously deposited in GenBank with the accession code AY701640. cDNA was created according to the methods described previously.⁶ Briefly, RNA was extracted from bone marrow and cDNA was produced by RT-PCR and cloned into the pCRII-TOPO® cloning vector (Invitrogen, Carlsbad, CA). The DNA was then subcloned into the pET12a vector (Novagen, Madison, WI).

Recombinant AL-12 and AL-103 proteins were expressed using the pET12a expression system in BL21 DE3 *E. coli* and purified as described previously.^{13,21} AL-12 was purified from the periplasmic space and AL-103 from solubilized inclusion bodies using 6 M urea immediately dialyzed into 10 mM Tris-HCl pH 7.4. Both proteins were purified using the HiLoad 16/60 Superdex 75 column on an AKTA FPLC (GE Healthcare, Piscataway, NJ) system. Pure protein was verified by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. The amino acid mutations were verified by Asp-N digestion and mass spectrometry analysis at the Mayo Proteomics Research Center. For further verification, amino acid analysis was performed by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

Circular Dichroism Spectroscopy (CD)

Protein secondary structure was monitored at 4 °C by Far UV CD (Jasco spectropolarimeter 810) from 260-200 nm as described previously.²⁰ Thermal denaturation experiments followed the ellipticity at 216 nm for AL-12 and 218 nm for AL-103 over a temperature range of 4-90° C and were analyzed to calculate the melting temperature (T_m , where 50% of the protein is unfolded).¹³ This was done as a quality control step to ensure that different protein batches present the same Far-UV CD spectrum and thermal denaturation properties, including complete refolding. Chemical denaturation experiments were carried out as described previously.^{10,13} $\Delta G_{\text{folding}}$ was determined from chemical denaturation for AL-12 and thermal denaturation for AL-103. The enthalpy (ΔH) was determined from the thermal denaturation data using the van't Hoff equation.³³

Analytical Size Exclusion Chromatography

The oligomeric state of the purified protein was determined using Bio Sil 125-5 HPLC size exclusion column on an AKTA FPLC (GE Healthcare, Piscataway, NJ). Gel filtration

molecular weight standards containing Thyroglobulin, Bovine gamma-globulin, Chicken ovalbumin, Equine myoglobin and Vitamin B₁₂ were used to calibrate the column (BioRad, Hercules, CA). The lyophilized standards were re-constituted with 0.5 mL deionized water, filtered and 100 μ L of this solution was used for injection onto the column. The column was calibrated with the standards at a flow rate of 0.2 mL/min. An elution buffer containing 0.05 M Na₂HPO₄, 0.05 M NaH₂PO₄, 0.15 M NaCl and 0.01 M NaN₃ was used at pH 6.8. A calibration curve was produced by plotting the logarithm of molecular weight standards as a function of their elution volume and determining the line of best fit. The elution volume for each standard peak was determined. The purified AL-103 was injected and eluted through the column at a flow rate of 0.2 mL/min in the same buffer as the standards. The apparent molecular weight of AL-103 was calculated using the line of best fit for the calibration curve.

Crystallization/X-ray Data Collection

Purified AL-12 and AL-103 proteins were concentrated to 923 μ M and 1.26 mM respectively, in 10 mM Tris-HCl buffer (pH 7.4). AL-12 crystals were obtained in hanging drops using vapor diffusion against 20% w/v polyethylene glycol 4000 and 0.2 M Li₂SO₄ in 0.1 M Tris-HCl buffer (pH 6.8), with a thin layer of 60% paraffin oil and 40% silicon oil at 22 °C. A 2 μ L aliquot of the protein solution was mixed with an equal volume from each reservoir. AL-12 crystals were soaked in 30% w/v Xylitol for cryoprotection using liquid N₂. AL-103 crystals were obtained in hanging drops using vapor diffusion against 15% w/v polyethylene glycol 8000 and 0.05 M magnesium acetate and 0.1 M sodium acetate in 0.1 M Tris-HCl buffer (pH 5.4) at 22°C. A 2 μ L aliquot of the protein solution was mixed with an equal volume from each reservoir. AL-103 crystals were cryoprotected with a 20% w/v Ethylene glycol solution using liquid N₂. Diffraction data was collected at 1.5241 nm on a home X-ray source, Rigaku/MSO 007 microfocus generator, with Osmic VariMax optics, Xstream²⁰⁰⁰ cryostream and a R-axis IV⁺⁺ detector. All data were collected at 100 °K. Table 2 summarizes the statistics for the crystallographic diffraction data collections and structural refinement.

Structure Refinement

Diffraction patterns for AL-12 and AL-103 were processed with Crystal Clear.³⁴ All structures were solved by molecular replacement with the κI O18/O8 structure (2Q20.pdb) using PHASER. Programs REFMAC5³⁵ and COOT³⁶ were used for structure refinement and model building. TLS (translational/libration/screw-rotational) parameters were used to model atomic displacements with one TLS domain set for each monomer within the asymmetric unit. The stereochemistry and the agreement between model and X-ray data were verified by COOT,³⁶ MOLPROBITY,³⁷ PROCHECK,³⁸ and SFCHECK³⁹. AL-12 and AL-103 had 0.00% Ramachandran outliers. AL-12 had 95.2% in favored Ramachandran orientations and AL-103 had 93.46%.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. VL structure

A) Topological Diagram of the protein structure for AL-12 and AL-103 adapted from Schiffer.⁴⁰ The two β -sheets of the domains have been separated. Residues that point towards the core are circled. CDR segments are highlighted in yellow. The β -strands have been encased within the arrows and are connected to their respective loops. AL-12 mutations are shown in red, AL-103 mutations in green.

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<-----FR1-----><-----CDR1-----><-----FR2-----><CDR2-><-----FR3-----><CDR3-><-----FR4----->
1   5   10  15  20  25  30 abcdef 35  40  45  50  55  60  65  70  75  80  85  90  95  100 105
      sssss sssss sssssss sssssss sss sssss sssssss sssssss sss sssss
VKI_018/08 DIQMTQSPSSLSASVGDRTITTCQASQDISN-----YLNWYQOKPKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDATYYCQYDNLP
AL-09      DIQMTQSPSSLSASVGDRTITTCQASQDIN-----YLNWYQOKPKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDATYYCQYDNLP--YTPGGGKLEIK JK2
AL-12      DIQMTQSPSSLSASVGDRTITTCQASQDITN-----HLNWYQOKPKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDATYYCQYDLP--QTPGGGKVEIK JK4
AL-103     DIQMTQSPSSLSASVGDRTITTCQASQDISN-----YLNWYQOKPKAPKLLIYDASNLETGVPSRFSGSGSGTDFTFTISSLQPEDATYYCQYHNLPR--YTPFGKLEIK JK2

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Figure 2.

Sequence Alignment of AL-12, AL-103 and the germline sequence κI O18/O8.

Protein sequence alignment of AL-12 and AL103 with their parent germline sequence κI O18/O8 highlights a diversity of somatic mutations within the framework regions. AL-12 has 8 mutations, 7 of which are non-conservative, whilst AL-103 has 4, all of which are non-conservative including a proline insertion after position 95, referred to as 95ProIns. As a comparison to our previous work, the sequence of AL-09 has been included for completeness. AL-09 has 7 mutations, 3 of which are non-conservative. The generally accepted VL domain topology according to Kabat is provided (<http://www.kabatdatabase.com/index.html>). Germline sequence determination done using VBase website, (<http://vbase.mrc-cpe.cam.ac.uk/>). Secondary structure is shown (s= β-strand).

Figure 3.

Mutation locations for AL-12 and AL-103.

Molecular model for the crystal structure of AL-12 and AL-103 are shown in A and B respectively. The mutational positions for each crystal structure are indicated in grey spheres and labeled on each molecular model. The model for both AL-12 and AL-103 depicts the expected β -sheet rich, greek-key motif, typical of the immunoglobulin fold. Part C shows the superposition of AL-12 (yellow) and the germline protein κ I O18/O8 (blue) highlighting that AL-12 adopts the canonical germline dimer interface. This is also true for AL-103 (not shown). The red arrows indicate the proline-40 and the proline-95 loops.

Figure 4.

Structural Perturbations in both AL-12 and AL-103 are subtle.

Upon superposition of the AL-12 or AL-103 it can clearly be observed that there are structural deviations in the region of proline 95. There is significant disruption to the backbone conformation in AL-103 in order to accommodate the proline insertion. Previous publications have shown similar small scale structural changes as those seen in the model structures for both AL-12 and AL-103^{18,10}. These deviations cause re-arrangements of the hydrogen bonding patterns within this loop region. Despite the high resolution of diffraction, completeness and redundancy for both models the poor density in this region is indicative of greater mobility of this loop.

Figure 5.

RMS comparison via Cα backbone superposition reveals larger areas of structural variability and instability.

Using RMSD to compare Cα deviations gives a visual aid to help represent the structural deviations observed upon superposition of AL-12 (top) and AL-103 (bottom) models with either κI O18/O8 (A, B) or AL-09 (C, D). The smaller difference scale seen in A and B, when compared to C and D show that both AL-12 and AL-103 share more structural similarities with κI O18/O8 than AL-09. A and C highlight the small structural deviations seen in the proline-40 region of the AL-12 model as well as those in the proline-95 region. B and D highlight the relatively larger structural deviation seen in AL-103 in order to compensate for the insertion.

The result of the insertion seems to be contained within the proline-95 loop and there are few knock-on consequences into the surrounding strands. The disruption represented here by high RMSD leads to hydrogen bond re-arrangements within the loops. This is significant as this directs hydrogen bonding away from the dimer interface.

Figure 6.

Electron density for CDR3 loop region of AL-12 and AL-103

Figure 5 shows the electron density map, scaled at 2.0 sigma, for the proline-95 region of both AL-12 and AL-103. Key features and mutations in this region are labeled. For AL-12, there are significant density breaks throughout the loop region. In an attempt to satisfy all $F_o - F_c$ multiple conformations were modeled for residue 96. The carbonyl group of proline-95 is modeled in both trans- and cis- conformations. The electron density breaks for AL-103 are less clear, but can be seen around the proline insertion. This insertion is accommodated to some extent by Tyr-96 being recruited further into the loop region to re-enforce intra-loop hydrogen bonding.

Table 1
Thermodynamics of restorative and reciprocal mutants

Protein	T _m (°C)	C _m (M)	ΔG _{folding} (kcal/mol)	ΔH (kcal/mol)
AL-09 [*]	41.1 ± 1.0	1.88 ± 0.07	-3.53 ± 0.28 [§]	-62.8 ± 1.0
AL-12 ^{**}	46.1 ± 0.2	2.3	-3.6	-94.9
AL-103	41.6 ± 0.5	ND	-4.43 ± 0.07 [#]	-71.1 ± 2.5
κI O18/O8 [*]	56.1 ± 0.2	3.98 ± 0.07	-6.12 ± 0.23	-95.7 ± 2.6

^{*} As reported previously¹¹

^{**} As reported previously¹⁵

[§] From chemical denaturation data at 4 °C

[#] From thermal denaturation data at 25 °C

Error shown is S.D. from at least three independent experiments.

Table 2
Crystallographic statistic table for AL-12 and AL-103

	AL-12	AL-103
Space group	P6 ₁ 22	P 4 ₁ 2 ₁ 2
Cell <i>a</i> , <i>b</i> , <i>c</i> (Å)	81.4, 81.4, 77.2	47.0, 47.0, 103.3
<i>a</i> , <i>b</i> , <i>g</i> (°)	90, 90, 120	90, 90, 90
Molecules in asymmetric unit	1	1
Resolution (Å)	77.15-1.79 (1.84-1.79)	103.14-1.57 (1.61-1.57)
Completeness (%)	99.5 (98.5)	95.5 (64.9)
Redundancy	3.6 (3.8)	16.9 (6.5)
Number of residues	107	109
Number of protein atoms	905	916
Number of solvent atoms	95	202
R _{merge}	0.036 (0.34)	0.071 (0.35)
<1/σ ₁ >	17.2 (3.3)	20.8 (4.0)
R _{work} (%)	19.29 (33.0)	19.01 (32.4)
R _{free} (%)	26.56 (42.8)	25.18 (42.7)
No. of Reflections	13,965 (994)	15,784 (763)
Average B-factor protein atoms	37.3	23.3
Average B-factor water molecules	60.4	36.4
R.m.s. Deviations		
Bond Length Å	0.026	0.023
Bond Angle (°)	1.967	1.880
Ramachandran Plot		
Most favored regions (%)	95.2	93.46
Outliers (%)	0.00	0.00

Values in parenthesis are that for the highest resolution binned data as shown.