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Recognition of antigens by nanobodies from structural and energetic viewpoints.

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Single-domain antibodies (VHHs or nanobodies) from camelids are considered next-generation therapeutic biologicals. Although they display a small size, their affinity and specificity for antigens rival those of antibodies of the IgG class. The question that we aim to clarify in this study is how such small antibodies are able to reach such high performance.

1 Introduction

Fragments of antibodies and other scaffolds of small size lacking the large Fc fragment are increasingly valid alternatives to the more classical IgG molecules because of their pharmacokinetic properties. Miniature single-domain antibodies from camelids (VHHs) are increasingly employed as next generation candidates because of their therapeutic potential. VHHs possess numerous advantages, including the ease to being modified, fused with other proteins, or in drug-conjugates. VHHs are not only valuable therapeutic molecules, but also can be employed as sensors for various applications, as tools in cell-biology and even as chaperons to facilitate crystallization.

One of the stunning properties of VHHs is their ability to specifically bind antigens with very high affinity despite their miniature size. By employing a long complementarity determining region 3 (CDR3), VHHs compensate for the limitations of their small size and tightly engage the target antigen with great specificity. This hypothesis was advanced in a previous structural study [1]. An important question that remains to be elucidated is how VHHs energetically recognize the antigen and in particular the individual contribution of residues located at the binding interface.

In this study we sought to explore the structural and thermodynamic basis of the recognition of a model VHH for its antigen. For that purpose, data from crystals of wild type and mutants of the VHH (termed D3-L11) in complex with its antigen hen egg lysozyme (HEL) were collected at high resolution at the Photon Factory.

2 Experiment

The VHH termed D3-L11 was prepared as previously reported [2] in *Escherichia coli*. The protein was purified by immobilized-metal affinity chromatography and size-exclusion chromatography in a buffer containing 20 mM TRIS-HCl, 150 mM NaCl, and 1 mM EDTA at pH 7.4. The antigen HEL was purchased from Wako Pure Chemical (Cat. No. 126-02671, Japan), and prepared in a buffer containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 (pH 7.4).

Crystallization of the complex between VHH and the antigen was obtained after purification of the complex in excess HEL by size exclusion chromatography in 20 mM TRIS-HCl and 100 mM NaCl (pH 8.0). Sample of the complex at 8-13 mg/mL was crystallized in a solution containing 100 mM sodium nitrate and 16% PEG-3350 (pH 8.0) or 100 mM lithium chloride and 18 % PEG-3350 for the complex containing WT or Y102A VHH, respectively. Crystals of unbound WT at 10 mg/mL were obtained in a solution of 100 mM Tris-HCl and 2.15 M ammonium sulfate (pH 7.0).

Data were collected at BL5A and AR-NW12 of the Photon Factory under cryogenic conditions (100 K). Data analysis, and structure determination and refinement were carried out using standard procedures. The resolution of the crystal structures ranged from 1.15 to 1.65 Å. Thermodynamic and affinity data were obtained by isothermal titration calorimetry and surface plasmon resonance.

3 Results and Discussion

The kinetic and energetic analysis of WT VHH and mutants of residues located at the antibody-antigen interface revealed several hot-spot residues (Figure 1). These are residues that carry a large contribution of the favorable binding energy [3]. We identified interfacial Tyr52 and Tyr102 as such hot-spot residues and determined the crystal structure of the complex of the mutant Y102A with HEL at high resolution. Despite of a large loss of binding energy (~5 kcal/mol), the structure of the complex did not change in any appreciable fashion

(Figure 2), suggesting a rigid binding recognition like that described in the classical lock-and-key mechanism. This hypothesis was further corroborated by showing that the unbound form of the WT antibody (resolution 1.15 Å) had essentially the same structure as that in the complex (not shown).

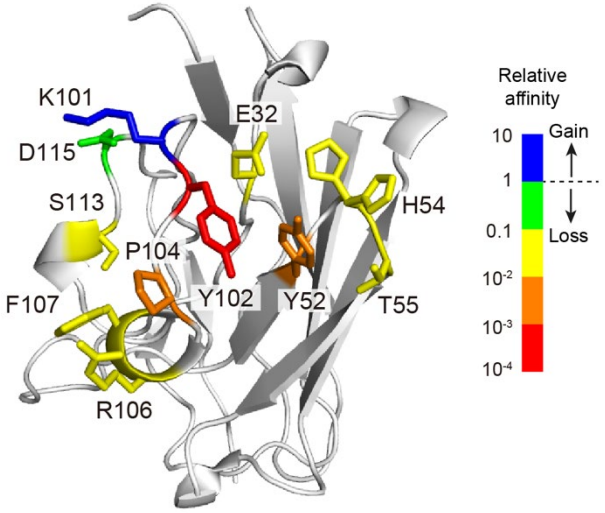

Fig 1: Mutagenesis analysis of VHH. Residues interacting with the antigen were mutated to Ala and their effect to binding was determined. Residues are colored according to the effect on binding, where red and blue indicate large and small contributions, respectively. Adapted from [2].

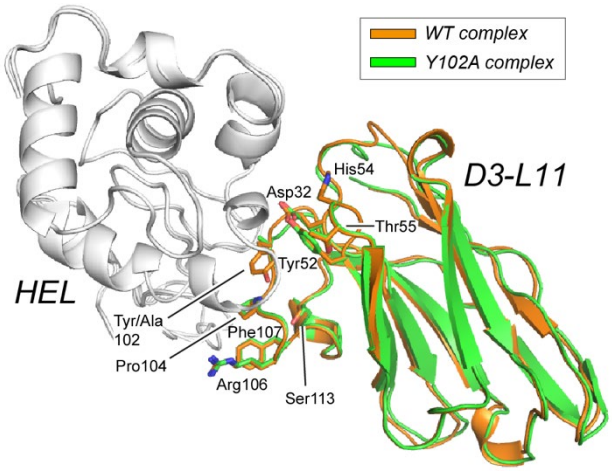

Fig. 2: Comparison of the crystal structure of complexes of WT VHH or its mutant Y102A with HEL. HEL, WT and Y102A are shown in gray, orange and green, respectively. Adapted from [2].

Overall our results indicate that VHH antibodies bind to the concave surface of antigens employing hot-spot residues in a mechanism that resembles that of the classical lock-and-key model (Figure 3).

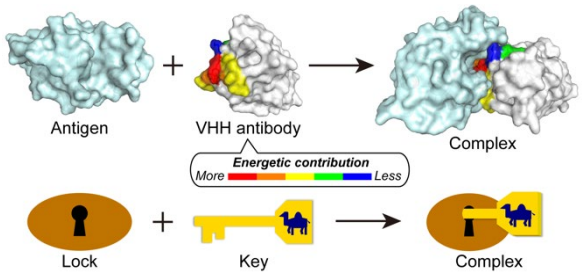

Fig. 3: Model of VHH-antigen intetaction.

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