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Light chain somatic mutations change thermodynamics of binding and water coordination in the HyHEL-10 family of antibodies

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ABSTRACT

Thermodynamic and structural studies addressed the increased affinity due to L-chain somatic mutations in the HyHEL-10 family of affinity matured IgG antibodies, using ITC, SPR with van't Hoff analysis, and X-ray crystallography. When compared to the parental antibody H26L26, the H26L10 and H26L8 chimeras binding to lysozyme showed an increase in favorable ΔG° of -1.2 ± 0.1 kcal mol^{-1} and -1.3 ± 0.1 kcal mol^{-1}, respectively. Increase in affinity of the H26L10 chimera was due to a net increase in favorable enthalpy change with little difference in change in entropy compared to H26L26. The H26L8 chimera exhibited the greatest increase in favorable enthalpy but also showed an increase in unfavorable entropy change, with the result being that the affinities of both chimeras were essentially equivalent. Site-directed L-chain mutants identified the shared somatic mutation S30G as the dominant contributor to increasing affinity to lysozyme. This mutation was not influenced by H-chain somatic mutations. Residue 30L is at the periphery of the binding interface and S30G effects an increase in hydrophobicity and decrease in H-bonding ability and size, but does not make any new energetically important antigen contacts. A new 1.2-Å structure of the H10L10–HEL complex showed changes in the pattern of both inter- and intra-molecular water bridging with no other significant structural alterations near the binding interface compared to the H26L26–HEL complex. These results highlight the necessity for investigating both the structure and the thermodynamics associated with introduced mutations, in order to better assess and understand their impact on binding. Furthermore, it provides an important example of how backbone flexibility and water-bridging may favorably influence the thermodynamics of an antibody–antigen interaction.

Somatic mutation of B-cell receptors, followed by clonal selection and expansion, is an essential vertebrate immune mechanism for developing highly specific and potent antibody response. Somatic mutations occur throughout the H- and L-chains. Mutations in the binding loops making up the complementarity determining regions (CDR) and in the supporting framework regions (FR) can have an important influence on binding affinity and specificity (Thielges et al., 2008; Torres and Casadevall, 2008; Weitkamp et al., 2005). Identification and thermodynamic characterization of somatic mutations which increase affinity and/or change specificity often provide important details on strategies that can be applied to other protein interactions (Chowdhury and Pastan, 1999; Dougan et al., 1998; Du et al., 2007; Kumagai et al., 2003; Lavoie et al., 1992; Sagawa et al., 2003; Thielges et al., 2008). This has direct relevance, but is not limited to, the strong interest in the therapeutic use of antibodies (Jain et al., 2007; Presta, 2006; Yan et al., 2008).

Identifying energetically favorable somatic mutations from sequence data or X-ray structures of complexes is a difficult challenge (Dixon et al., 2002; Welfle et al., 2003). The structure of an antibody–antigen complex can identify non-bonded contacts, hydrogen bonds, salt bridges and localization of hydrophobic and hydrophilic residues that are potentially important to binding to the antigen. The effects of mutations or their relative importance can still be very difficult to predict or interpret from structures

Abbreviations: CDR, complementary determining region; HEL, hen egg white lysozyme; SPR, surface plasmon resonance; ITC, isothermal titration calorimetry; FR, framework residues; RU, response unit; IgG, immunoglobin G; SASA, solvent accessible surface area.

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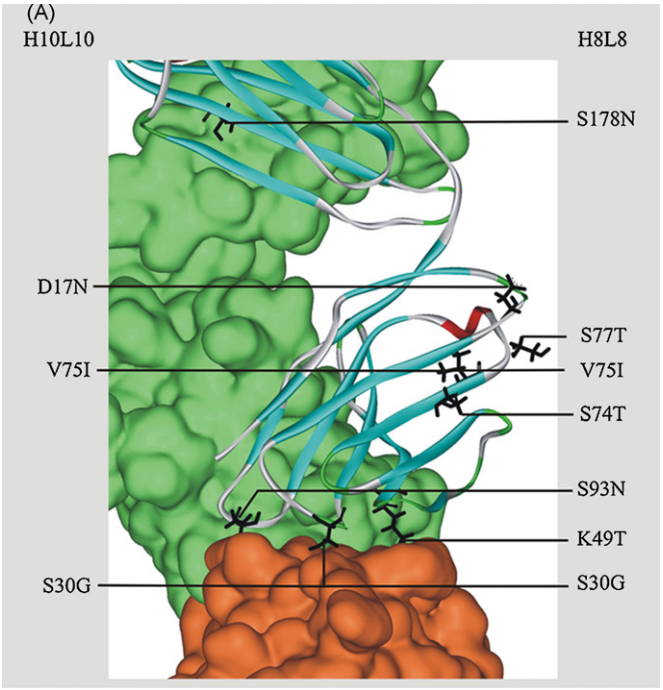

(B)

Position	17	30	49	74	75	77	93	178
H26L26	D	S	K	S	V	S	S	S
H26L10	N	G	K	S	I	S	S	S
H26L8		G	T	T	I	T	N	N

Fig. 1. (A) Spatial relationship of somatic mutations in the structure of H26L26 in complex with HEL (PDB ID: 1NDM). H-chain (green) and lysozyme (orange) are displayed as van der Waals surfaces. The L-chain backbone is modeled in ribbon form. Somatic mutations are highlighted in stick form (black). (B) Sequence summary of amino acid changes resulting from somatic mutation compared with H26L26 (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article).

alone. Thermodynamic characterization is essential for the unambiguous identification of those residues that provide favorable energetic contributions to antigen binding.

Three independently derived affinity matured IgG antibodies (HyHEL-26, HyHEL-10, and HyHEL-8) recognize similar coincident epitopes on the antigen HEL. All use the same V_L germline gene (IgkV23) with identical V–J junctions. The only differences between the L-chain sequences are from somatic mutations (Fig. 1) (Lavoie et al., 1999). HyHEL-26 is the least evolved (early secondary), lowest affinity, and least cross reactive in this family. Both HyHEL-10 and HyHEL-8 are late secondary hyper-immune IgGs and exhibit higher affinity and greater cross reactivity with HEL mutants. Together, these three antibodies represent a useful model of an *in vivo* engineering response for improving affinity for a protein antigen.

Many H-chain somatic mutations in these affinity-matured antibodies have been characterized (Newman et al., 1992; Shiroishi et al., 2007; Smith-Gill et al., 1984, 1982), but much less is known about the L-chain somatic mutations. Here we identify functionally significant L-chain residues and detail their structural impact relative to two-step thermodynamic analysis using a new 1.2 Å structure of the HyHEL10 Fab–HEL complex. The impact of heavy chain context is also explored. A single serine to glycine substitution in CDR1 at the periphery of the binding interface significantly increases affinity and alters the binding thermodynamics but does not result in large perturbations in the structure of the complex.

1. Materials and methods

1.1. Protein expression, refolding and purification

Hen egg white lysozyme (Worthington Biochemicals, NJ, USA) was further purified using gel filtration chromatography. The isolation of IgGs and construction of the corresponding recombinant Fab fragment expression system have been described elsewhere (Lavoie et al., 1992; Newman et al., 1992). Fab L- and H-chains were expressed separately in BL21(DE3) (EMD Biosciences, San Diego, CA) cells as inclusion bodies at 37 °C. Expression, isolation and purification of inclusion body protein followed previously outlined procedures (Li et al., 2003). Refolded protein was concentrated 20-fold, buffer exchanged (10 mM Tris, 2 mM EDTA, pH 8.0) using a TTF filter (Pall Corp, East Hills, NY), and loaded onto a DEAE Sepharose Fast-flow column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Sample was eluted with a linear gradient (0–400 mM NaCl over 3 column volumes). Soluble Fab was the first protein to elute and corresponding fractions were pooled, concentrated, and further purified using a Superdex 75 HR gel filtration column (GE Healthcare Bio-Sciences, Uppsala, Sweden). Purity of samples was assessed using SDS-PAGE and elution profiles from gel filtration. For all subsequent analysis, samples were extensively dialyzed against HBS buffer (10 mM HEPES, 150 mM NaCl, 2 mM EDTA, pH 7.4). Fabs of wild type H26L26, and chimeric H26L10 and H26L8 were successfully refolded from bacterial inclusion bodies as judged by the ability to selectively bind HEL using SPR. Contamination from misfolded soluble aggregates and chain homodimers was eliminated using ion exchange and gel filtration chromatography. An unstable off-pathway intermediate from the refolding process formed a soluble metastable aggregate that bound more strongly to the ion exchange column than the properly folded Fab. This species bound to the antigen lysozyme in SPR assays, albeit with much reduced affinity (data not shown), precluding use of an HEL-affinity column for purification purposes. This species was eliminated in the void volume of the gel filtration column. Total yield from the refolding process, measured as the amount of soluble, properly folded protein, ranged from 5 to 45% of the starting material. Stock protein samples were stored at 4 °C in HBS buffer in the presence 0.02% sodium azide until further use. Stability of samples under these storage conditions was evaluated after six months and there was no degradation, aggregation or change in kinetics as judged by gel filtration chromatography and SPR (data not shown).

1.2. Site-directed mutagenesis

Sequence changes to the H26L26 L-chain corresponding to the naturally occurring somatic mutations were introduced individually using the Quikchange II Mutagenesis kit (GE Healthcare Bio-Sciences, Uppsala, Sweden). All mutations were verified by sequencing (DNA Sequencing Laboratory NCI-Frederick, MD, USA).

1.3. Isothermal titration calorimetry

All purified protein samples were prepared for ITC analysis by co-dialyzing extensively against HBS. Measurements were made using a VP-ITC Microcalorimeter (MicroCal LLC, MA, USA) at 15, 25 and 35 °C. Experiments consisted of injections of 8 μL aliquots of 0.10–0.25 mM HEL into 10–25 μM Fab in the ITC cell. This was preceded by a single initial 3 μL injection to minimize impact of diffusion at the ligand/protein interface of the syringe tip during thermal equilibration. Experiments were repeated using dialysis buffer to replace the antibody in the cell. These 'blank' measurements were subtracted from the ligand-into-Fab titration results.

The integrated interaction heat values were fitted to the one-binding site model using Microcal Origin software, yielding the