Structure-based engineering of a monoclonal antibody for improved solubility

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Protein aggregation is of great concern to pharmaceutical formulations and has been implicated in several diseases. We engineered an anti-IL-13 monoclonal antibody CNTO607 for improved solubility. Three structure-based engineering approaches were employed in this study: (i) modifying the isoelectric point (pI), (ii) decreasing the overall surface hydrophobicity and (iii) re-introducing an N-linked carbohydrate moiety within a complementarity-determining region (CDR) sequence. A mutant was identified with a modified pI that had a 2-fold improvement in solubility while retaining the binding affinity to IL-13. Several mutants with decreased overall surface hydrophobicity also showed moderately improved solubility while maintaining a similar antigen affinity. Structural studies combined with mutagenesis data identified an aggregation ‘hot spot’ in heavy-chain CDR3 (H-CDR3) that contains three residues (96FHW100m). The same residues, however, were found to be essential for high affinity binding to IL-13. On the basis of the spatial proximity and germline sequence, we reintroduced the consensus N-glycosylation site in H-CDR2 which was found in the original antibody, anticipating that the carbohydrate moiety would shield the aggregation ‘hot spot’ in H-CDR3 while not interfering with antigen binding. Peptide mapping and mass spectrometric analysis revealed that the N-glycosylation site was generally occupied. This variant showed greatly improved solubility and bound to IL-13 with affinity similar to CNTO607 without the N-linked carbohydrate. All three engineering approaches led to improved solubility and adding an N-linked carbohydrate to the CDR was the most effective route for enhancing the solubility of CNTO607.

Keywords: antibody/N-glycosylation/protein engineering/solubility/structure-based design

Introduction

Successful development and launch of a new protein therapeutic is dependent on the selection of a protein candidate with good activity and biophysical properties. The biophysical properties of candidate molecules have implications for development of therapeutics relating to purification, formulation, pharmacokinetics, immunogenicity and dosing regimens. For biopharmaceuticals, low solubility of therapeutic proteins renders formulation development more difficult and may lead to poor bio-distribution, undesirable pharmacokinetics behavior and immunogenicity in vivo (Kerns and Di, 2003). Indeed, several recent reports on computational and traditional biophysical methods have described new strategies to characterize and improve the biophysical properties of therapeutic candidates (Jespers et al., 2004; Famm and Winter, 2006; Lienqueo et al., 2006; Demeule et al., 2007; Jacobs et al., 2009).

The intrinsic properties of proteins such as size, hydrophobicity, electrostatics and charge distribution play important roles in protein solubility (Bagby et al., 2001; Mosavi and Peng, 2003; Andrews et al., 2005; Strickler et al., 2006; Trevino et al., 2007). Aggregation or precipitation is driven by protein self-association, which is often the result of weak molecular interactions. Such interactions are a function of the solution environment and include factors such as pH, ionic strength, temperature and co-solutes. Recognizing the importance of solubility, a variety of strategies have been employed to improve protein solubility including site-specific mutagenesis (Fowler et al., 2005; Pawar et al., 2005; Swencki-Underwood et al., 2008) and stabilizing peptide fusions (Lee et al., 2005). Covalent modification and conjugation of proteins by pegylation or hyperglycosylation frequently used to enhance pharmacokinetics and in vivo half-life also often result in higher solubility (Wiecek et al., 2007; Veronese and Mero, 2008).

CNTO607 is a potent neutralizing anti-IL-13 monoclonal antibody selected using Morphosys HuCAL phage display technology (Rothe et al., 2008). An examination of the original complementarity-determining region (CDR) sequences from phage display selection indicated the presence of a consensus N-linked glycosylation site (s3NSSs) in heavy-chain CDR2 (H-CDR2). Expression of the mAb in mammalian cell cultures may thus lead to glycosylation at this site. This could potentially lead to additional heterogeneity of the final therapeutic mAb. To improve homogeneity, the potential glycosylation site was removed by replacing N53 with an aspartic acid, a residue commonly encoded at this position in the corresponding germline genes (Vh3). The resulting antibody retained high affinity for IL-13, although the antibody exhibited low solubility and aggregation near neutral pH. In an effort to improve the biophysical properties of the antibody, we employed three protein-engineering strategies: (i) altering the isoelectric point (pI); (ii) decreasing overall surface hydrophobicity and (iii) introducing an N-linked carbohydrate within H-CDR2 to disrupt a suspected mechanism of aggregation based on structural studies. We demonstrate that shielding an aggregation-promoting hydrophobic patch on the surface of the antibody with a carbohydrate
moiety provided dramatic improvement in solubility while the other strategies yielded only incremental improvements.

**Materials and methods**

**Reagents**

Human recombinant IL-13 was purchased from R&D System (Minneapolis, MN, USA). Sialidase A was obtained from SelectinBio (Pleasanton, CA, USA). Trifluoroacetic acid (TFA), EDTA disodium salt and 8 M guanidine hydrochloride stock solution were purchased from Thermo Scientific Inc. (Rockford, IL, USA). Acetonitrile was purchased from Honeywell International Inc. Burdick & Jackson (Muskegon, MI, USA). Dithiothreitol (DTT) was purchased from Bio-Rad (Hercules, CA, USA). Iodoacetamide was purchased from Sigma-Aldrich Inc. (St Louis, MO, USA). Ultrapure 1 M Tris-hydrochloride stock solution was purchased from Invitrogen (Carlsbad, CA, USA). Lysyl endoproteinase C mass spectrometry grade was purchased from Wako Chemicals USA, Inc. (Richmond, VA, USA).

**Engineered antibodies with germline mutations**

The sequence of the light chain (LC) of CNT0607 was compared with germline sequences in the IMGT database (Lefranc et al., 2009) using BLAST (Rangwala and Karypis, 2007). The closest matching germline gene for LC *vβ_3h* (*V_l 3*) was chosen based on the overall sequence identity. Residues in the framework region were then mutated to their counterparts in the chosen germline gene.

**Construction of expression plasmids**

Gene-specific primers were designed and synthesized based on the heavy-chain (HC) V-region of CNT0607. The primers contained flanking HindIII and EcoRI restriction enzyme sites for cloning into the Lonza pEE GS (glutamine synthetase) vector that contains human IgG1 constant regions. CNT0607 HC expression plasmid was designated as p3401. The LC V-region of CNT0607 was cloned into the pEE GS vector that contains human lambda constant regions. CNT0607 LC expression plasmid was designated as p3408.

The plasmids, p3401 and p3408, were used as templates to generate different variants either on CNT0607 HC or on CNT0607 LC. Various mutations, as listed in Table I, were introduced using a QuickChange site-directed mutagenesis kit (Stratagene, Cat# 200522).

**Expression and purification**

CNT0607 used in these studies is a fully human IgG1λ monovalent antibody against human IL-13 (Centocor R&D) produced from an SP2/0 stable cell line. The mutant antibodies were transiently co-expressed by HC and LC plasmids in either HEK293E or CHO cells. Antibody variants were expressed transiently in 1 l volume HEK293E or CHO transfections. We did not observe cell-line specific, HEK293E or CHO, differences in antibody solubility among the CNT0607 variants. For each transfection, in separate tubes, 150 mg each of LC and HC DNA and Lipofectamine 2000 (Invitrogen Cat# 11668027) were diluted in 20 ml OptiMem media. The mixtures were incubated at room temperature for 5 min before combining the contents of both tubes and incubating for additional 20 min at room temperature. The DNA/Lipofectamine/OptiMem complex was added to the cells drop-wise directly into media and cells were placed in the incubator for 24 h. The transfection media was replaced with 293SFMMII production media (Invitrogen Cat# 11686029) 24 h later. The conditioned media were harvested for purification 6 days later, after the transfections were set up. Antibodies were purified from the harvested media using MahSelect SuRe resin (GE Healthcare, Cat# 17-5438-03). The purity of the antibodies was analyzed by SDS–10% polyacrylamide gel electrophoresis (PAGE) according to Laemmli (1970).

**Isoelectric-focusing gel analysis**

To determine the pI values of antibodies, isoelectric-focusing (IEF) gel electrophoresis was performed. Protein samples were analyzed with Novex Pre-Cast Vertical pI 3-10 IEF Gels (Invitrogen). Samples comprised of 2–5 mg of proteins were loaded on the gel. The detailed procedures were conducted according to the manufacturer’s protocol.

**Solubility measurements**

To determine the solubilities of antibodies, experiments were carried out using the ultrafiltration method (Haire and Blow, 2001; van Reis and Zydney, 2001; Moore and Kery, 2009). Antibodies in PBS buffer were added to Vivaspin ultrafiltration spin columns with 30 000 MWCO (Vivascience Ltd.) at

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**Table I. Sequence summary for anti-IL-13 monoclonal antibody variants**

<table>
<thead>
<tr>
<th>Light Chain</th>
<th>Heavy Chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequencea</td>
<td>1</td>
</tr>
<tr>
<td>CNT0607</td>
<td>SYELT........ISGTQAED</td>
</tr>
<tr>
<td>Mab II</td>
<td>SYLV.........ISIVEAGDE</td>
</tr>
<tr>
<td>Mab III</td>
<td>SYELT........ISGTQAED</td>
</tr>
<tr>
<td>Mab IV</td>
<td>SYLV.........ISIVEAGDE</td>
</tr>
<tr>
<td>Mab V</td>
<td>SYELT........ISGTQAED</td>
</tr>
<tr>
<td>Mab VI</td>
<td>SYLV.........ISIVEAGDE</td>
</tr>
<tr>
<td>Mab VII</td>
<td>SYLV.........ISIVEAGDE</td>
</tr>
</tbody>
</table>

*aResidue numbered annotations were identified according to Chothia structure-based numbering scheme (Chothia et al., 1998; Teplyakov et al., 2009).*
room temperature. The 15-ml spin columns were inserted to the swing bucket rotor in Eppendorf Centrifuge 5804R and centrifuged at speed 3000×g. Once the volume was reduced to 2 ml, supernatant was transferred to a 2-ml spin column and centrifuged at speed 4000×g for 20 min. When the volume was reduced to 500 µl, the sample was transferred to a Vivaspin 500 spin column and centrifuged at 15 000×g until precipitation was observed using an Eppendorf 5415R centrifuge. After spinning down the precipitation, the supernatant was equilibrated at room temperature overnight. After another centrifugation step to remove the precipitation, the supernatant was collected and the antibody concentration in solution was determined after appropriate dilution by measuring absorbance at 280 nm using a Nanodrop (Thermo Scientific Inc., Rockford, IL, USA). If the concentrations reach more than 100 mg/ml without visible precipitation, further concentration was stopped and the protein concentration was determined as described.

**Binding affinity determination by Biacore analysis**

Surface plasmon resonance experiments were performed using a Biacore 3000 optical biosensor (Biacore AB, Uppsala, Sweden; currently part of GE Healthcare). The experiments were performed at 25°C in D-PBS buffer containing 3 mM EDTA and 0.005% surfactant P20. To analyze the interaction of the antibodies with IL-13 (R&D systems, Inc.), a capture surface was generated by covalent coupling of goat antihuman IgG Fc fragment (Jackson ImmunoResearch Laboratories, Inc.) to a CM-5 sensor chip. The anti-human Fc fragment-specific antibody was diluted into 10 mM sodium acetate buffer pH 4.5 (Biacore AB) and coupled to the carboxymethylated dextran surface of the CM-5 chip [~3000 response units (RU)] using the manufacturer's instructions for amine-coupling chemistry. The remaining reactive groups on the surface were deactivated using ethanolamine–HCl. To perform kinetics experiments, ~100 RU of anti-IL-13 antibodies were captured on this surface followed by injection of IL-13 at various concentrations (typically 0.10–5 nM) for an 8-min period at 30 µl/min. This was followed by buffer flow for 10 min to 1 h to monitor binding dissociation. The capture surface was regenerated using 100 mM phosphoric acid, followed by injection of running buffer.

Double reference subtraction of the data was performed to correct for buffer contribution to the signal and instrument noise using the Scrubber software (Myszka, 1999) version 1.1 g (BioLogic Software) for referencing. After this initial data processing, kinetic analysis of the data was performed using the BIAevaluation software, version 4.0.1 (Biacore AB) assuming a simple 1:1 binding model.

**Hydrophobic interaction chromatography**

The procedure used to measure the antibody retention time in hydrophobic interaction chromatography (HIC) is similar to one described previously (Lienqueo et al., 2006; To and Lenhoff, 2007a) with some modifications. The experiments were performed on a Beckman-Coulter HPLC system equipped with a System Gold® 126 Solvent Module gradient pump, a System Gold® 508 Autosampler with a 200-µl injection loop and System Gold® UV detector of eluted protein. A TSK-GEL® Ether-5PW column (7.5 × 0.75 cm) made of stainless steel and 10 µm diameter particles (PN 08641, Tosoh Bioscience, LLC, Montgomeryville, PA, USA) was equilibrated at room temperature with 80% 2 M ammonium sulfate in PBS using a flow rate of 0.5 ml/min. Samples were diluted to ~100 µg/ml and 100 µl was injected. The gradient used for each isocratic elution was 80% 2 M ammonium sulfate/20% PBS in 35 min.

After each isocratic run, the column was washed with 5–10 column volumes of PBS and then re-equilibrated with five column volumes of buffer containing the appropriate ammonium sulfate concentration prior to the next sample injection.

**Characterization of N-glycosylation**

Antibody samples (~1 mg/ml) were brought into 20 mM Tris–HCl buffer, pH 7.5, and digested with sialidase A (at 1:100 enzyme to substrate ratio) at 37°C for 24 h (Raju and Scallon, 2007). The desialylated antibody samples were purified using a protein A affinity column and subjected to IEF gel analysis. pls were determined by applying the antibodies to Novex® IEF Gels (Invitrogen, CA, USA) contain 5% polyacrylamide and following the manufacturer’s instructions.

The glycosylation in Mab VII was verified using HPLC-Qq/TOF mass spectrometry (Agilent 1100 coupled with a Waters Q-TOF MS). Peptide mapping of Mab VII was performed as described previously (Dorai et al., 2009) with some modifications. Briefly, 200 µg of the mAb was desalted, dried and reconstituted with 150 µl of 6 M guanidine hydrochloride, 50 mM Tris–HCl, 5 mM EDTA, pH 8.0. The sample was reduced with 1.5 µl of 1.0 M DTT at 37°C for 1 h and alkylated with 3.0 µl of 1 M sodium iodoacetamide at room temperature in the dark for 1 h. The reaction was quenched by the addition of 1 µl of 1.0 M DTT. The reduced and alkylated sample was then diluted with 750 µl of 50 mM Tris–HCl, pH 8.0, and digested with Lys-C (enzyme/protein = 1:25) (w/w) for 4 h at 37°C. The reaction was quenched by the addition of 5 ml of TFA, and the resultant peptides were resolved by reverse-phase HPLC using a narrow bore C18 column (Vydac, 218TP5215, 2.1 mm × 150 mm) and a gradient of acetonitrile in 0.1% TFA. The chromatography was performed on an Agilent HPLC (model 1100) with a capillary pump and a diode array detector. Absorbance was monitored and collected at 214 and 280 nm. An online LTQ-Orbitrap mass spectrometer (Thermo Electron) with an electrospray source was used in-line after the UV detector to collect mass spectra of the intact peptide as well as to fragment the peptides for sequencing (MS/MS analysis).

**Thermal stability analysis**

Thermal stability experiments were carried out in PBS. Antibodies were introduced into the sample chamber of a Nano DSC calorimeter (Calorimetry Sciences/TA Instruments) at concentrations of 0.5–1.0 mg/ml. The corresponding PBS dialysate used in the sample preparation was introduced into the reference chamber. Each mAb-buffer pair was heated linearly from 20°C to 100°C at a fixed pressure of ~3 atm, while differential power was monitored. In addition, PBS was added to both sample and reference chambers and scans were collected for baseline subtraction using the same procedure as for the protein samples.

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Results

The solubility of a protein depends strongly on the pH of the solution. We use the term solubility here to define the maximum protein concentration that can be achieved before precipitation is observed. The highest concentration of CNTO607 in PBS buffer at pH 7.2 is \( \approx 13 \text{ mg/ml} \). To evaluate the antibody in a biologically relevant pH environment, we focused on improving CNTO607 solubility in this buffer.

The effect of pH

The range for pH of CNTO607 was determined by IEF gel analysis to be 7.4–7.5 (Fig. 1). The multiple bands observed in the gel are typical of antibodies due to charge heterogeneity introduced by the variability of the N-linked glycosylation at N\(_{297}\) in the Fc region (Nimmerjahn and Ravetch, 2008; Raju, 2008). Owing to the close proximity of the pH to 7.4, it was considered unlikely that these residue changes would impact antigen binding. Therefore, a variant, Mab II, with mutations of E\(_3\) \( \rightarrow \) V and 77GTQAE\(_{81} \rightarrow \) 77RVEAG\(_{81}\) in framework regions 1 and 3, respectively. Thus, a CNTO607 variant with such changes would have a net charge increase of 2. On the basis of the crystal structures of the Fab fragment of CNTO607 and its complex with IL-13 (Teplyakov et al., 2009), it was considered unlikely that these residue changes would impact antigen binding. Therefore, a variant, Mab II, with mutations of E\(_3\) \( \rightarrow \) V and 77GTQAE\(_{81} \rightarrow \) 77RVEAG\(_{81}\) in the LC was constructed.

![IEF gel analysis of CNTO607 and Mab II](image)

Fig. 1. IEF gel analysis of CNTO607 and Mab II. Ten microliters of pH 3-10 markers (Serva) was loaded on the gel. Positions of pH markers are indicated at left. Each lane contained 4–10 \( \mu \text{g} \) of protein.

The effect of hydrophobicity

Hydrophobic interactions have long been recognized as major contributors to high-affinity interactions between macromolecules. Given the high affinity of CNTO607 to IL-13 (18 pM), it is not surprising that the CDR loops in CNTO607 contain a number of hydrophobic residues that form a prominent hydrophobic ridge (Fig. 2A). Both the LC and the HC contribute to this structural feature. Clusters of such hydrophobic residues on the surface of proteins tend to favor non-specific interactions that lead to aggregation (Nasreen et al., 2006). On the basis of the crystal structure of the Fab fragment of CNTO607 bound to IL-13, most of these hydrophobic residues are involved directly in IL-13 binding (Teplyakov et al., 2009). Nevertheless, we attempted to reduce the surface hydrophobicity of the CDR loops in CNTO607 by site-directed mutagenesis.

A number of diverse light-chain CDR3 (L-CDR3) sequences were discovered during the phage display affinity maturation process, indicating tolerance in the L-CDR3 region. On the basis of the crystal structure of the complex between CNTO607 Fab and IL-13, the principal antibody residues that contact IL-13 are located in H-CDR3, L-CDR1 and L-CDR2, whereas H-CDR1 and L-CDR3 make very few

<table>
<thead>
<tr>
<th>Mab ID</th>
<th>KD (pM)</th>
<th>Solubility (mg/ml)</th>
<th>( \Delta T_r ) (min)</th>
<th>( T_m ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNTO607</td>
<td>18.4 ( \pm ) 1.9</td>
<td>13.3</td>
<td>0.0</td>
<td>63.7</td>
</tr>
<tr>
<td>Mab II</td>
<td>23.3 ( \pm ) 5.6</td>
<td>29.1</td>
<td>( -0.3 )</td>
<td>ND</td>
</tr>
<tr>
<td>Mab III</td>
<td>70.8 ( \pm ) 0.9</td>
<td>25.4</td>
<td>( -1.5 )</td>
<td>ND</td>
</tr>
<tr>
<td>Mab IV</td>
<td>29.1 ( \pm ) 0.8</td>
<td>12.4</td>
<td>( -1.6 )</td>
<td>ND</td>
</tr>
<tr>
<td>Mab V</td>
<td>7.6 ( \pm ) 0.5</td>
<td>29.2</td>
<td>( -2.8 )</td>
<td>69.4</td>
</tr>
<tr>
<td>Mab VI</td>
<td>&gt;45 000</td>
<td>&gt;164</td>
<td>( -6.5 )</td>
<td>64.9</td>
</tr>
<tr>
<td>Mab VII</td>
<td>22.2 ( \pm ) 6.6</td>
<td>&gt;110</td>
<td>ND</td>
<td>61.9</td>
</tr>
</tbody>
</table>

The equilibrium dissociation constant KD is calculated from the ratio of \( k_{d}/k_{a} \) using a Biacore analysis. Data were obtained from a series of protein concentrations. The retention time, \( T_r \), of antibodies was obtained in HIC. \( \Delta T_r = T_r \) (variant) – \( T_r \) (Mab I). ND, not determined. \( T_m \) was determined by DSC on Fv transition.
contacts with IL-13 (Teplyakov et al., 2009). Hence, we replaced the L-CDR3 with more hydrophilic sequences, resulting in Mab III, IV and V (Table I). Biacore data showed that these CNTO607 variants bind to IL-13 with affinities similar to that of CNTO607. The structural and binding results suggested that these antibody variants would likely recognize a very same epitope. The hydrophobicity of these antibodies was evaluated by HIC. The retention time of a protein by HIC is determined by protein surface hydrophobicity as well as protein size (Lienqueo et al., 2006; To and Lenhoff, 2007a). When two proteins have virtually the same size and similar structures, the less hydrophobic protein will elute earlier on an HIC column. Although Mab III, IV and V eluted earlier on an HIC column, suggesting reduced surface hydrophobicity (Table II), the solubilities of Mab III (∼25 mg/ml), Mab IV (∼12 mg/ml) and Mab V (∼29 mg/ml) were not improved dramatically. Thus, the HIC retention time does not correlate linearly with solubility. These data also indicated that the L-CDR3 hydrophobicity was not critical for CNTO607 aggregation.

Identification of the aggregation 'hot spot'

In the analysis of the crystal structure of the Fab fragment of CNTO607, it was observed that a nearly symmetrical tetramer was formed as a result of crystal-packing contacts (Fig. 3A; Teplyakov et al., 2009). In this tetramer, an aromatic amino acid residue triad 99FHW100a in H-CDR3 of a Fab molecule was nestled against the elbow region of a symmetry-related Fab molecule (Fig. 3B). These three residues located near the center of the hydrophobic ridge occupy the center of the antigen-recognition site found in the three-dimensional structure of the Fab:IL-13 complex (Fig. 2B; Teplyakov et al., 2009). The potential contribution of these residues to CNTO607 aggregation was explored by mutating them all to alanine residues, resulting in Mab VI (99FHW100a → 99AAA100a). This triple alanine mutant was found to be
highly soluble in PBS buffer (>160 mg/ml) and significantly less hydrophobic as indicated by a 6.5 min reduction in the HIC elution time (Table II). However, not surprisingly, Mab VI no longer binds to IL-13 with high affinity (Table II). This result pinpointed the aromatic triad in the H-CDR3 sequence as a ‘hot spot’ for both aggregation and interaction with IL-13.

A variable region N-glycan to prevent aggregation

The crystal packing of the Fab (Fig. 3A and B) suggested a potential mechanism of aggregation. If this tetrameric packing exists in solution, albeit at low affinity, it would likely lead to large clusters of mAbs at high protein concentration due to the bivalency of the mAb. Thus, we sought to disrupt this potential specific mAb–mAb polymerization while keeping the hydrophobic ‘hot spot’ in the H-CDR3 sequence intact for antigen binding. By careful examination of the structures of Fab607 and Fab607/IL-13 complex, we concluded that D53 of CNTO607 H-CDR2 was adjacent to the aromatic triad and exposed to the solvent, but not directly involved in antigen binding so that placement of an N-linked carbohydrate moiety at D53 might block the mAb–mAb interaction (Fig. 2B). Therefore, D53 of CNTO607 H-CDR2 was mutated to N in combination with the pI mutations in the HC (Table I). The resulting molecule, Mab VII, was generated and characterized for solubility and ligand binding.

Upon concentration, Mab VII was found to be soluble to >110 mg/ml in PBS buffer (Table II). Although the upper limit of the solubility could not be determined because of the quantity limitation, it was obvious that Mab VII exhibited far superior solubility when compared with Mab II. Biacore analysis indicated that Mab VII bound to IL-13 with affinity comparable to CNTO607 and Mab II (Table II). Mab VII was analyzed further to determine whether the N-glycosylation site was occupied using analytical methods including SDS–PAGE, IEF gel analysis combined with sialidase A treatment and peptide mapping using mass spectrometry. The HC of Mab VII showed distinctively higher molecular weight than that of CNTO607 in SDS–PAGE gel under reducing condition (Fig. 4A), consistent with an additional N-linked carbohydrate moiety. The relatively narrow band on the gel also suggested that the N-glycosylation site was almost fully occupied. Although the IEF gel of CNTO607 showed multiple bands due to charge heterogeneity in the N-linked glycosylation in the Fc region, there were many more bands for Mab VII consistent with additional heterogeneity due to the new glycosylation in the Fv region. Upon treatment with sialidase A, which specifically removes the sialic acids on glycans, Mab VII showed a significant increase in pI (Fig. 4B) whereas CNTO607 shows little difference (data not shown). The insensitivity of CNTO607 to sialidase A treatment is consistent with previous reports that the N-linked glycosylation in the Fc region is minimally sialylated (Scallon et al., 2007). The large change observed for Mab VII suggested that the N-linked glycosylation in the Fv of Mab VII was significantly sialylated, analogous to the variable region N-linked carbohydrate in Erbitux (Qian et al., 2007). The peptide mapping study led to the conclusion that residue N53 was indeed glycosylated and that the carbohydrate primarily consisted of a core-fucosylated, tri-antennary structure with fucose (1), galactose (2–3), and N-acetylneuraminic acid (0–3) heterogeneity (Fig. 5, top panel). On the basis of peak areas from the HPLC chromatogram, it was estimated that ~96% of the antibody population had carbohydrate attached to N53 (Fig. 5, bottom panel).

Taken together, the analytical results demonstrated that an N-linked carbohydrate existed in the H-CDR2 loop of Mab VII. This carbohydrate moiety prevented aggregation of the antibody while allowing it to interact with the antigen with high affinity.

The effect of thermal stability

Poor thermal stability can affect the solubility of proteins and lead to aggregation (Ewert et al., 2003; Jespers et al., 2004; Riechmann and Winter, 2006; Christ et al., 2007; Famm et al., 2008; Hmila et al., 2008). We sought to determine whether low thermal stability contributed to the low solubility of some of the variants by measuring the melting temperature. A typical differential scanning calorimetry (DSC) profile for an antibody contains multiple transitions, corresponding to the unfolding processes of CH2 domain, the Fab fragment and CH3 domain, respectively (Garber and Demarest, 2007). Sometimes not all the transitions are resolved. The Fab fragment transition signal is typically the highest of the transition signals (Garber and Demarest, 2007). The sequences of the constant domains are invariable and isotype-dependent. Their unfolding transition temperatures are therefore less variable for antibodies of the same isotype. In contrast, the variable domain sequences can be very diverse, leading to a wide range of Fab unfolding transition temperatures from one antibody to another.

The thermal stability of CNTO607 and a number of the variants was evaluated by DSC (Table II). Figure 6A shows the DSC profiles of CNTO607, the Fab fragment of CNTO607 and IgG1-Fc. With the IgG1-Fc sample, we observed two transitions at 70°C and 82°C, which correspond to the CH2 and CH3 domains, respectively (Garber and Demarest, 2007). Fab607 (described in Teplyakov et al., 2009) yielded two transitions at 65°C and 77°C, corresponding to the Fv and CH1 domains, respectively. CNTO607 yielded ~4 transitions that generally aligned with the Fab and IgG1-Fc transitions. Therefore, we assigned the transitions near 63°C, 70°C, 76°C and 83°C to the Fv, CH2, CH1 and CH3 domains, respectively. Figure 6B shows the DSC profiles of CNTO607, Mab V, Mab VI and Mab VII. All of the antibodies in this study possessed the human IgG1 isotype and therefore had identical constant domains. The Fab fragment melting temperatures ranged from 61.9°C to 69.4°C. No correlation was observed between the melting temperatures and the solubility, albeit they are within a relatively small range. Both Mab V and Mab VII are highly soluble antibodies yet their melting temperatures were at the high and low end of the Tm range, respectively.

Discussion

In the work presented here, we successfully engineered a low solubility monoclonal antibody into more soluble antibodies while maintaining high affinity to the antigen. Three strategies were devised to improve the solubility: (i) modifying the pl, (ii) decreasing the overall surface hydrophobicity and (iii) re-introducing an N-linked carbohydrate moiety in the
H-CDR2 sequence. All three strategies had various degrees of success in improving solubility. Two of the variants, Mab VI and Mab VII, were highly soluble. However, Mab VI, with greatly reduced hydrophobicity, lost binding affinity to IL-13. Mab VII, on the other hand, is a highly soluble and potent antibody with elevated pI and the additional N-linked glycosylation in H-CDR2.

Changing the pI is known as a valid approach for increasing protein solubility (Tan et al., 1998). At a pH near the pI of a protein, the net charge on the protein is close to zero and the attractive electrostatic forces due to the dipole moment of the protein dominate protein–protein interactions. This often leads to non-specific aggregation of the molecules and results in low solubility. Away from the pI, the molecules are positively or negatively charged and the resulting repulsive forces promote higher solubility. Engineering the pI to improve protein solubility has been successfully carried out for an scFv, in which the pI was lowered from 7.5 to a predicted 6.1 by an addition of five glutamate residues at the C-terminus (Tan et al., 1998). This resulted in a 150-fold
increase in the solubility of the scFv. The pI change we were able to achieve in Mab II was small (0.4 pH units) in comparison, hampered by the desire to maintain human germline sequences to avoid immunogenicity. One can expect that additional changes in pI without sequence constraints may result in more significant improvement in solubility.

Hydrophobicity is well known to be involved in protein aggregation. Unfolded or partially unfolded proteins tend to form aggregates due to exposure of the hydrophobic interiors to the aqueous solvent (Patra and Udgaonkar, 2007; Ashbaugh and Hatch, 2008). Protein surface hydrophobic patches are a source of aggregation at high protein concentrations (Mosavi and Peng, 2003). Reducing surface hydrophobicity by substitution with hydrophilic residues proved to be an effective strategy for improving solubility in several cases (Dale et al., 2003; Mosavi and Peng, 2003; Derewenda, 2004). For example, replacement of surface-exposed hydrophobic residues with arginine in ankyrin repeat proteins and in the subtilisin family of proteases significantly improved their solubility at physiological pH (Shirai et al., 1997; Mosavi and Peng, 2003).

The hydrophobicity of a protein can be measured in different ways. HIC is a technique used to purify and characterize proteins (To and Lenhoff, 2007a, b). Compared with reverse-phase chromatography, which uses highly hydrophobic surfaces and harsh eluents, HIC employs less hydrophobic surfaces and milder protein–matrix interactions. Thus, proteins purified by HIC are more likely to retain their native conformations and biological activities (Lienqueo et al., 2006; To and Lenhoff, 2007a). In this work, we used HIC retention time to provide a relative ranking of the overall surface hydrophobicity. We found that a reduction in overall hydrophobicity, measured by the HIC retention time, sometimes yielded more soluble molecules but was not a reliable predictor for solubility.

We succeeded in engineering a highly soluble and potent antibody by adding a consensus N-glycosylation site into the H-CDR2 loop, paving the way toward high-concentration formulations. Hyperglycosylation (Sinclair and Elliott, 2005), similar to pegylation (Veronese and Mero, 2008), has been shown to improve properties such as molecular stability, solubility, increased in vivo biological activity and reduced immunogenicity for therapeutic proteins. Antibodies are natural glycoproteins with N-linked carbohydrates attached to N297 in the Fc region. Many antibodies also have variable region-associated oligosaccharides. In Kabat’s database, ~18% of the variable region sequences contain a potential N-linked glycosylation site in HCs. Approximately 15–25% of the Fab fragments and 15% of the LCs isolated from human myeloma proteins were found to contain N-linked oligosaccharides (Spiegelberg et al., 1970). Erbitux, a human/mouse chimeric therapeutic antibody derived using hybridoma technology, has an occupied N-linked glycosylation site within framework 3 of the HC variable region (Qian et al., 2007). Variable region glycosylation is known to play an important role in affinity, specificity, half-life, and immunogenicity of several antibodies (Wright et al., 1991; Coloma et al., 1999; Rudd et al., 1999). In many cases, carbohydrates in the variable regions are found to modulate affinity (Wallick et al., 1988; Wright et al., 1991; Co et al., 1993; Khurana et al., 1997; Leibiger et al., 1999). In a previous report, the presence of an N-linked carbohydrate placed in the same position as in Mab VII increased the affinity for dextran by 10–50-fold (Wright et al., 1991).CNT0607 was derived from the HuCAL Gold Fab phage display library, in which the consensus N-glycosylation sites were present in the V-regions (Rothe et al., 2008). In the latest HuCAL Platinum library, these N-glycosylation motifs have been largely eliminated (http://www.morphosys.com) to avoid potential negative glycosylation effect on affinity and heterogeneity when antibodies are produced in mammalian cell cultures. Glyco-engineering in antibody CDR sequences remains an uncommon practice due to the high risk of impeding antigen binding. To our knowledge, this is the first report that intentionally adding an N-linked carbohydrate in a CDR resulted in a highly soluble antibody without any significant effect on affinity.

In our case, the introduction of the single carbohydrate moiety in the H-CDR2 loop served to inhibit self-interactions. On the basis of the crystal structure, a bulky carbohydrate moiety attached to this site was predicted to protrude outward with minimal perturbation to the structure. Owing to the single-point attachment, the carbohydrate moiety apparently has sufficient flexibility not to interfere with the interaction with the antigen. Our work demonstrated that variable region hyperglycosylation can be a viable approach to improve antibody solubility. Although this approach has received little attention in the past, we believe that it may have wider applicability in antibody engineering than previously thought, especially when antigen interaction and aggregation ‘hot spot’ overlap. In a typical antibody–antigen binding system, it was shown that less than half of the potentially antigen contacting CDR residues are used in the binding interactions (MacCallum et al., 1996). Some of the remaining CDR residues are, in principle, available for
attaching carbohydrates that do not interfere with antigen binding. It is conceivable that this may be more feasible with small antigens. With detailed structural knowledge for the antibody–antigen complex, attachment sites can be rationally selected. In the absence of such structural information, a number of sites could be tested according to modeled structures of the antibody. The additional heterogeneity introduced by the variable region glycosylation may be limited by utilizing glycol-engineered cell lines (Li et al., 2006).

Although protein aggregation is usually considered to involve non-specific interactions, the interaction for CNT0607 involves a specific aromatic triad in H-CD3R. Our limited success in increasing solubility with the pI and LC hydrophobic mutants is likely due to the retention of the limited success in increasing solubility with the pI and LC hydrophobic mutants is likely due to the retention of the bivalency and lead to precipitation at high concentrations. Further studies are on-going to test the hypothesis.

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