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Investigation of antibody disulfide reduction and re-oxidation and impact to biological activities

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ABSTRACT

Disulfide reduction in therapeutic monoclonal antibodies can occur during cell harvest operations as a result of cell breakage. Understanding these product quality changes and manufacturers' ability to control them would likely be of concern to regulatory bodies. To study the biological impact of disulfide reduction, mAbs, including IgG2κ, IgG2λ, IgG1κ, and IgG1λ forms, were partially reduced with dithiothreitol (DTT). Samples generated had approximately 10% or 50% intact molecules as determined by nrCE-SDS. Similar to the type of partial reduction obtained during uncontrolled harvest operations, DTT reduced antibodies were free from sulfur-linked adduct, such as attached cysteine. These partially reduced materials were incubated under physiological (blood-mimicking) redox conditions *in vitro* to follow the fate of the interchain cysteines. Within 8 h, the original disulfide bonds reformed. For mAbA, an IgG2κ, the initial re-oxidized state favored the IgG2-A disulfide isoform, which then underwent conversion over time to other isoforms. Reduced material was fully active. Results suggest that the type of disulfide reduction would have minimal impact to safety or efficacy. Antibody re-oxidation rates were found to be in the order of IgG2κ < IgG2λ < IgG1κ and IgG1λ, the same order as previously determined reduction rates.

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1. Introduction

Recombinant monoclonal antibodies (mAbs) are prominent therapeutic proteins, with IgG1 and IgG2 isotypes the most commonly used [1–4]. A structural feature of an antibody is the interchain disulfide bond, which links its light chains (LCs) to heavy chains (HCs) and its two HCs together to form a covalent H_2L_2 quaternary complex [5]. Disulfide bonds play a significant role in protein stability and folding and can also impact structure and function. Often, protein stability is decreased when a disulfide bond is reduced or eliminated [6–8]. In a mammalian cell culture system for large scale therapeutic antibody production (*e.g.*, CHO), all of the disulfide bonds (both inter- and intrachain) of the antibody are correctly paired before the assembled antibody is secreted into the cell culture fluid (CCF). Reduction of antibody interchain disulfide bonds during manufacturing operations has recently been observed, and has been an area of concern for regulatory bodies [9–12]. This phenomenon is observed when extending the time that the antibody remains in the CCF or harvested CCF (HCCF). Reduction is caused by the release of intracellular reducing components as a result of excessive cell lysis generated by shearing forces.

Since manufacturers' ability to control partial reduction would likely be of concern to regulatory bodies, we had studied the effect of process parameters and intrinsic molecule properties on the extent of reduction [13]. A "worst case" reduction model of cell culture extract was generated by mechanically shearing and sparging the resultant slurry with nitrogen to simulate the anaerobic environment of the commercial scales. CCFs from several cell lines were compared. We found that CCFs from some cell lines did not reduce antibodies, even under fully sheared conditions. Differences in reducing power in fully sheared CCF samples must be due to differences in cellular components. In addition, the degree of reduction was dependent on the antibody class and LC type. The trend of reducibility follows the order IgG1λ > IgG1κ > IgG2λ > IgG2κ. This order matched a bench-scale model using a thioredoxin/thioredoxin reductase regeneration system, and DTT reduction sensitivity. Thus, product attributes, cell lines, and process parameters all contributed to the extent of antibody reduction during production.

Understanding these product quality changes and their biological impact were the goals of this study. Partially reduced mAbs, IgG2κ, IgG2λ, IgG1κ, and IgG1λ, were produced by treatment with DTT, and were characterized by non-reduced (nr) chip based CE-SDS, limited protease digestions followed by RP-HPLC-tandem MS,

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and non-reducing LysC peptide mapping. To study the biological impact of partial reduction, partially reduced mAbs were incubated under physiological (blood-mimicking) redox conditions *in vitro* (15 μM cysteine, 250 μM cystine, phosphate buffered saline, pH 7.4 at 37 °C). These samples were then analyzed for interchain reduction levels by microchip based nrCE-SDS. For IgG2s, changes to the disulfide isoform distribution were followed by RP-HPLC and non-reducing peptide mapping methods.

2. Materials and methods

2.1. Materials

Recombinant human monoclonal antibodies mAbA [IgG2κ], mAbB [IgG2κ], mAbC [IgG2λ], mAbD [IgG1κ], and mAbE [IgG1λ] were expressed in Chinese hamster ovary (CHO) cells and purified by standard manufacturing procedures [14] at Amgen Inc. (Thousand Oaks, CA). Tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) solution, 1.0 M, pH 7.5, was produced by Teknova (Hollister, CA). N-ethylmaleimide (NEM) and fabRICATOR (IdeS) were purchased from Sigma Aldrich (St. Louis, MO). TFA (0.1%) in acetonitrile (ACN), TFA (0.1%) in water, 20% sodium dodecyl sulfate (SDS), sodium phosphate (NaPi) monobasic monohydrate, sodium phosphate dibasic, 7-hydrate, sodium chloride (NaCl), 10 N sodium hydroxide (NaOH), and 5 N hydrochloric acid (HCl) were J.T. Baker reagents (Phillipsburg, NJ). Lysyl endopeptidase (Lys-C) was from Wako Chemicals USA, Inc. (Richmond, VA). HyClone DPBS, Float-A-Lyzer dialysis devices, NAP-10 columns (GE Healthcare, Little Chalfont, UK), trifluoroacetic acid (TFA), and DTT were obtained from Thermo Fisher Scientific (Waltham, MA). Iodoacetamide (IAM), guanidine hydrochloride (GuHCl) L-cystine, L-cysteine and sodium azide (NaN_3) were from MP Biomedical (Irvin, CA). DTNB (5,5′-dithio-bis 2-nitrobenzoic acid) was from Invitrogen (Carlsbad, CA). HT Protein Express Chips and HT Protein Express Reagent Kit are products of Caliper Life Sciences (Hopkinton, MA).

The Zorbax 300SB-C8 (2.1 mm × 50 mm, 3.5 μm particles, 300 Å) was manufactured by Agilent Technologies (Santa Clara, CA). ACQUITY UPLC BEH300 C4 Columns (2.1 mm × 150 mm, 1.7 μm particle), and BEH Phenyl (2.1 mm × 150 mm, 1.7 μm particles) column were purchased from Waters Corporation (Milford, MA).

2.2. Reduction by DTT

Partially reduced mAbs, with approximately 10% or 40% intact form (determined by nrCE-SDS), were generated by incubating mAbs at the concentration of 8 mg/ml with DTT in 50 mM Tris–HCl, pH 7.5 at room temperature for 10–50 min. For IgG2 molecules, 3 mM DTT was used, while IgG1 molecules needed 0.6 mM DTT. To remove reductant, the sample was buffer exchanged using an NAP-10 column into 10 mM sodium acetate (NaOAc), pH 5.0 and stored at −70 °C.

For reduction kinetic studies, aliquots of the reduction reaction mixture were taken at multiple time points and quenched with NEM to a final concentration of 25 mM.

2.3. In vitro dialysis

To mimic blood thiol redox conditions, a flow-through dialysis system with cysteine and cystine was used [15]. Briefly, this system uses a chromatography system to mix concentrated cysteine and cysteine stocks to achieve a final concentration of 15 μM cysteine and 250 μM cystine. Partially reduced antibodies are placed in a dialysis device and placed inside the dialysis chamber to mimic blood redox conditions. A thermally jacketed chromatography column serves as a dialysis chamber. This system is capable of maintaining a stable redox level over long periods. These results are used to predict the fate of partially reduced antibody *in vivo*.

2.4. Non-reduced chip based CE-SDS

Microchip based capillary electrophoresis-sodium dodecyl sulfate (CE-SDS) was performed on a Caliper LC90 or GXII (Caliper Life Sciences) under non-reducing condition. The assay was described previously [16], with the following modifications. A total of 5 μL of sample at approximately 1 mg/ml was mixed with 30 μL of HT Protein Express Sample Buffer (Caliper Life Sciences) and 60 μL 8 mM NEM in water (final concentration of 5 mM NEM). The samples were incubated at 75 °C for 10 min prior to the analysis by LC90 or GXII with the "HT Protein Express 200" program.

2.5. IdeS digestion-mAbA

Partially reduced and re-oxidized mAbA samples were treated with 1 mM NEM final concentration for 30 min at RT before digesting with the protease IdeS (fabRICATOR, Genovis Inc) and reducing with DTT. IdeS cleaves IgGs below the hinge. After disulfide reduction of the sample the Fc/2 (single chain of Fc), LC, and Fd (single chain including variable region of HC, constant region 1 of HC with 4 cysteines in the hinge region) can be analyzed [17]. The fragments are each approximately 25 kDa in size; they are well separated by RP-HPLC. Using Agilent MSD Time of Flight (TOF) MS, the amount of reduced cysteine was quantified by measuring NEM addition (+ 125 Da for each NEM labeled cysteine).

Percent labeled LC (only one interchain disulfide bond) was calculated by dividing percent of labeled (B) by the sum of percent labeled and unlabeled (A). Percent labeled for Fd was calculated as an average of percent labeled of the five disulfide bonds. In the formula below, B, C, D, E, and F, are percent labeled for 1, 2, 3, 4 and 5 NEM labeled forms, respectively. A is percent unlabeled.

$$\text{LC\%Labeled} = \frac{B}{A + B}$$

$$\text{Fd\%Labeled} = \frac{B \times 1 + C \times 2 + D \times 3 + E \times 4 + F \times 5}{(A + B + C + D + E + F) \times 5}$$

Six micrograms of the antibody was digested with 60 units of IdeS enzyme in 30 μL reaction buffer (50 mM sodium phosphate, 150 mM NaCl, pH 6.6) at 37 °C for 30 min. The disulfide bonds were reduced by mixing 30 μL of the digested sample with 30 μL of a denaturing buffer (8 M guanidine hydrochloride, 0.1 M Tris, pH 8.3) and 3 μL of freshly prepared 1.0 M DTT (in Milli-Q Water) at 55 °C for 30 min.

2.6. LC/MS

Non-reducing RP-HPLC was performed on an Agilent 1100 HPLC System with a Poros R1/10 column (4.6 mm × 50 mm). Mobile phase A was 0.1% TFA in water. Mobile phase B was 0.1% TFA in 90% ACN/10% water. The gradient was: 30% B at 0 min; 50% B at 30 min; 90% B at 46 min; then hold at 90% B for 4 min. The column temperature was maintained at 75 °C and with a flow rate of 0.2 ml/min. Five to fifteen micrograms of protein was injected for each analysis. Chromatogram was monitored with 214 nm UV spectra and MS. MS was performed with a LCT Premier, an electrospray time-of-flight mass spectrometer (Waters, Milford, MA).

2.7. Physiological redox incubations using flow-through dialysis system

To mimic blood thiol redox conditions, a flow-through dialysis system with cysteine and cystine was developed, as previously

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