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Prediction of the Hydrogen Peroxide–Induced Methionine Oxidation Propensity in Monoclonal Antibodies

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ABSTRACT

Methionine oxidation in therapeutic antibodies can impact the product's stability, clinical efficacy, and safety and hence it is desirable to address the methionine oxidation liability during antibody discovery and development phase. Although the current experimental approaches can identify the oxidation-labile methionine residues, their application is limited mostly to the development phase. We demonstrate an *in silico* method that can be used to predict oxidation-labile residues based solely on the antibody sequence and structure information. Since antibody sequence information is available in the discovery phase, the *in silico* method can be applied very early on to identify the oxidation-labile methionine residues and subsequently address the oxidation liability. We believe that the *in silico* method for methionine oxidation liability assessment can aid in antibody discovery and development phase to address the liability in a more rational way.

Introduction

Monoclonal antibodies (mAbs) are an important class of therapeutic molecules for treating various human conditions. To be a viable therapeutic candidate, the mAb needs to be stable against degradation under manufacturing processes and storage. Generally, mAb molecules have multiple pathways and mechanisms of degradation which often lead to a variety of degraded products. Oxidation of methionine residues in mAbs is one such form of degradation. Methionine oxidation can occur both *in vivo* and *in vitro*. Factors that lead to reactive oxygen species such as light exposure, trace metals, and chemical oxidants can drive methionine oxidation, and these factors can be present during protein production, purification, formulation, fill-finish, and storage.[1,2] For example, low levels of peroxides found in polysorbate, a commonly used surfactant in liquid formulations, can drive methionine oxidation during storage.[3]

Oxidation of methionine residues in an antibody can impact its potency by: reducing the binding to its intended target, reducing the half-life, or impacting effector function. Therefore, methionine residues that are critical for the potency of an antibody are often critical quality attributes, and their oxidation levels are required to be controlled to maintain the drug product's potency. Ideally, a drug candidate with a minimal number of these critical methionine residues is more desirable from manufacturing perspective than a candidate with large number of these residues. To select such a candidate, antibodies can be engineered and screened so that only those that are free of these liabilities are progressed for further development. Among the multitude of methionine residues that are present in an antibody sequence, generally, only a handful of residues are prone to oxidation. Classifying each of the methionine residues into "oxidation labile" versus "stable" is ideally required to efficiently focus the protein engineering and screening efforts. Although the oxidation propensity under "real-life" conditions are not known during early stages of discovery development, forced or stressed conditions (e.g., accelerated oxidation induced by hydrogen peroxide) are routinely used during the developability/manufacturability assessment to identify these labile residues.

In silico predictive methods that can identify these oxidation-labile methionine residues by using the antibody sequence as an input offer certain advantages over experimental methods. Predictive methods allow the identification of a methionine residue as a labile residue without the need for any material generation and thus can provide early guidance to protein engineering efforts and enable judicious resource allocation for a wet lab assessment of only high-risk methionine residue–containing molecules. Similar *in silico* strategies for prediction of other chemical and physical degradation liabilities in antibodies have been proposed.[4-9] For predicting methionine oxidation liability, a few *in silico* have been

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published.[4,10-14] However, due to a lack of consistent experimental data sets (i.e., methionine oxidation data generated under identical experimental conditions such as buffer, temperature, oxidizing agent concentration, etc.), a conclusive validation of these *in silico* tools, which could enable routine application of the *in silico* tool for engineering and screening process, for ranking methionine residues across different molecules for their oxidation propensity, is lacking. For example, methionine oxidation data for different proteins used by Chennamsetty et al.[4] were collected under nonidentical conditions and, therefore, the authors could only validate the ranking of methionine residue oxidation rates on the same protein instead of different proteins. Similarly, a conclusive validation of the water coordination number (WCN) model by Chu et al.[10] could not be performed since the model was applied to only one protein.

In this article, we present the oxidation rate of 54 methionine residues over 8 mAbs collected under identical experimental conditions. These 54 methionine residues cover a broad range of oxidation rates and provide a robust validation of the *in silico* model. Similar to the previous work, the focus is on the experimental and computational oxidation of methionine by hydrogen peroxide with a future goal of validating the *in silico* model for other oxidizing agents.

Materials and Methods

Experimental Method

Materials

All the fully human mAbs were recombinantly produced using house in mammalian expression systems and purified. All antibodies were formulated at pH 5.2 with acetate buffer. Hydrogen peroxide was purchased from Sigma Aldrich. Solution of 5% H_2O_2 was freshly prepared by dilution in MilliQ water.

Oxidation of Methionine Residues

The mAbs were diluted to approximately 4 mg/mL concentration in acetate buffer at pH 5.2. Two microliters of 5% H_2O_2 was added to 100 μL of antibody solution, and the solution was allowed to incubate at 25°C. Following each incubation period, the oxidation reaction was quenched by transferring the samples into an equilibrated Zeba® spin desalting column (purchased from Thermo Fisher and equilibrated with acetate buffer by following the manufacturer's directions), and the samples were collected by centrifuging the columns at 1500 × *g* for 2 min. Recovery following buffer exchange with Zeba columns was typically better than 90% for mAbs.

Mass Spectrometry

Oxidized samples (100 μg) were denatured by diluting to a final concentration of 0.9 mg/mL into a buffer containing 0.25-M Tris, 7.5-M guanidine-HCl, 2-mM EDTA, pH 7.5. Disulfide bonds were reduced by incubating these denatured samples for 30 min in 10-mM dithiothreitol. Subsequently, the reduced samples were carboxymethylated at room temperature for 30 min by adding iodoacetic acid to 20 mM. Samples were then buffer-exchanged into 0.1-M Tris, 0.05-M methionine, pH 7.5 by using Zeba® spin columns. Trypsin digestion was achieved by using a ratio of 1:10 (enzyme:sample) and incubating at 37°C for 30 min. The reaction was terminated by addition of 20% trifluoroacetic acid to a final concentration of 1.3% (v/v)

The digested samples were analyzed by liquid chromatography tandem-mass spectrometry (MS/MS). The liquid chromatography MS/MS system consisted of a Waters ACQUITY® UPLC system connected in-line to a Thermo Scientific Q Exactive Hybrid Quadrupole-Orbitrap mass spectrometer. A reversed-phase HPLC column (Waters ACQUITY® UPLC BEH130 C18 1.7 μm, 2.1 × 150 mm) was used to separate the peptides with the column temperature at 50°C and mobile phase A: 0.1% formic acid (v/v) in water and mobile phase B: 0.1% formic acid in acetonitrile. The gradient (hold %B at 1.0% for 5 min, then 1%-10%B in 1 min and 10%-36%B in 67 min) was performed at 0.25 mL/min. About 30 μg of samples was injected. Owing to instrument availability, data for some samples were acquired using a Thermo Q Exactive, whereas a Thermo Q Exactive Plus was used to analyze other samples. The instruments were operated in positive mode using data-dependent acquisition (top 6 for Q Exactive, top 4 for Q Exactive Plus). MS1 resolution was set to 35,000 for the Q Exactive and 70,000 for the Q Exactive Plus. The scan range for both instruments was 300-2000 m/z. MS2 were acquired at 17,500 resolution for both instruments using normalized collision energy of 25. Automatic gain control was set to 1E6 and maximum IT was set to 200 ms for both instruments.

Relative quantitation was performed using Skyline v. 3.6.0.10493.[15] For each molecule, a workbook was created containing unoxidized, that is, native and oxidized versions of all methionine-containing tryptic peptides. Total area of unoxidized and oxidized peptides was determined by extracting ion chromatograms of 2 charge states per peptide (3 isotopes per charge state). Acceptance criteria for each peptide charge state were mass accuracy $\leq$5 ppm and isotopic dot product $\geq$0.9. For some peptides, particularly at t = 0 time points, isotopic dot products for one of the charge states were less than 0.9. In these instances, only a single charge state was used for relative quantitation. Modification percentages were calculated by dividing the total area of the oxidized peptide by the sum of total areas from the oxidized and unoxidized peptides.

Oxidation Rate Modeling

The oxidation kinetics of a methionine residue was modeled using the following pseudo first-order rate law:

$$\mathrm{M} \rightarrow \mathrm{M}^{*} \tag{1}$$

where, 'M' is the unoxidized methionine residue and 'M*' is the oxidized methionine residue. Hence, the oxidation rate becomes

$$\frac{d[\mathrm{M}]}{dt} = -\mathrm{k}[\mathrm{M}] \tag{2}$$

where [M] is the molar concentration of a particular unoxidized methionine residue and k is the pseudo first-order rate constant. Solving the above equation leads to the following equation:

$$\left(\frac{[\mathrm{M}]}{[\mathrm{M}]_{\mathrm{t}=0}}\right) = \mathrm{e}^{-\mathrm{kt}} \tag{3}$$

We re-express this equation in terms of fraction of a particular methionine residue that is oxidized by time 't', that is, $([\mathrm{M}]/([\mathrm{M}]_{\mathrm{t}=0} + [\mathrm{M}^{*}]_{\mathrm{t}=0}))$ and add correction for cases where a minor amount of methionine oxidation is present at t = 0:

$$(1 - \text{fraction oxidized at t} = \text{'t'}) = (1 - \text{fraction oxidized at t} = 0) \times \mathrm{e}^{-\mathrm{kt}} \tag{4}$$