Modeling the Oxidation of Methionine Residues by Peroxides in Proteins

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ABSTRACT: We report the use of molecular modeling to predict the oxidation propensity of methionine residues in proteins. Oxidation of methionine to the sulfoxide form is one of the major degradation pathways for therapeutic proteins. Oxidation can occur during production, formulation, or storage of pharmaceuticals and it often reduces or eliminates biological activity. We use a molecular model based on atomistic simulations called 2-shell water coordination number to predict the oxidation rates for several model proteins and therapeutic candidates. In addition, we implement models that are based on static and simulation average of the solvent-accessible area (SAA) for either the side chain or the sulfur atom in the methionine residue. We then compare the results from the different models against the experimentally measured relative rates of methionine oxidation. We find that both the 2-shell model and the simulation-averaged SAA models are accurate in predicting the oxidation propensity of methionine residues for the proteins tested. We also find the appropriate parameter ranges where the models are most accurate. These models have significant predictive power and can be used to enable further protein engineering or to guide formulation approaches in stabilizing the unstable methionine residues. © 2015 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

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INTRODUCTION

Oxidation of proteins has been shown to contribute to aging in organisms as well as several diseases such as Parkinson's and Alzheimer's.[1–4] Oxidation is a major concern for therapeutic proteins as well because it can impact the safety and efficacy of the molecule.[5–8] Oxidation can occur at a number of residues within a protein including methionine, cysteine, tryptophan, tyrosine, and histidine residues.[9,10] Among these, methionine is often the most susceptible residue for oxidation.[11–14]

Methionine oxidation is categorized as site specific when it occurs through metal catalysts or nonsite specific when it occurs through peroxides or photo-oxidation.[12,15–17] In the current report, we focus on methionine oxidation because of peroxides, which is a major source of oxidation within therapeutic systems. These peroxides can come from the contaminants within excipients or degradants of surfactants used in formulation, or from containers or tubing such as those used in intravenous infusion systems.[12,18,19] Peroxide is also used as a sanitizing agent for manufacturing isolators. Forced oxidation through peroxides such as hydrogen peroxide followed by peptide mapping and Liquid Chromatography–Mass Spectrometry is the most common procedure used by formulation scientists to understand methionine oxidation liability in therapeutic proteins.[15,17] However, these experimental approaches are both tedious and expensive.

Proteins typically have multiple methionine residues each of which may oxidize to a different degree. Oxidation of these methionines has been shown to be affected by their solvent exposure and protein conformational stability.[20–22] Therefore, if the protein structure is known, modeling based on structure could help in identifying the methionine residues most prone to oxidation. Even if the protein X-ray structure is not available, a homology modeled structure could be built in most cases. This is especially true for mAbs (monoclonal antibodies), which are a major class of therapeutic proteins. The accuracy of these homology models is increasing with each passing year.[23] Thus, if a reliable model for oxidation can be built based on protein structure, it could help protein development by lowering the costs of liability assessment along with giving faster results. Identifying the most oxidation-prone methionines will enable researchers to mitigate these liabilities either through direct mutation in cases where binding activity is not impacted, or other strategies such as formulation optimization.[7] Therefore, development of a reliable validated model that can predict the most oxidation-prone methionines and rank them according to their oxidation propensity will be extremely useful.

The solvent-accessible area (SAA) is the most commonly used model to predict the propensity for methionine oxidation. In this model, the solvent-exposed surface area of the side chain of a methionine residue is calculated.[24] A static protein conformation (X-ray or homology modeled structure) is typically used in calculating SAA because of its simplicity and ease of use. However, a model based on static conformation could be error prone depending on the specific conformation used. The

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accuracy of SAA model could be improved by doing a simulation average, but it is not known exactly how much improvement in oxidation prediction can be obtained through simulation. Another model called the 2-shell water coordination number was introduced by Chu et al.[20,25–28] to estimate the relative rate of oxidation of methionine residues. This model is based on the proposed water-mediated mechanism for methionine oxidation because of peroxides in the pH range 2–8.[28] Using *ab initio* calculations, Chu et al.[28] showed that the water molecules near the sulfur atom help stabilize the transition state of the oxidation reaction through specific interactions with the partial charges that occur in the transition state. On the basis of this observation, they developed the 2-shell model that counts the average number of water molecules within two water coordination shells from the sulfur atom in the methionine residue. They showed that the 2-shell model correlates better than SAA model when compared with the experimental rate of oxidation of methionine residues. However, their study was limited to only a couple of model proteins (GCSF and hPTH).[20,26] There is also a question of whether the SAA model accuracy improves if only the sulfur atom that undergoes oxidation is used as the basis for SAA instead of the whole side chain. Thus, there is a need to rigorously evaluate the performance of these different SAA-based models along with the 2-shell model in a broad set of proteins including therapeutic candidates, and to find the optimal parameter ranges where the models are most accurate.

In the current report, we implement the 2-shell model and several SAA-based models over seven model proteins as well as three therapeutic candidates currently in development. The model proteins selected were those for which experimental oxidation data were available in the literature. The therapeutic candidates were selected for which there is a measurable difference in the experimental rates of oxidation for different methionines within each protein. In each case, we compare the relative oxidation propensity results from the different models with the experimental data. We test whether the models can (1) predict the most oxidation prone methionines, (2) rank the relative oxidation of different methionines, and (3) give quantitative or semiquantitative agreement with the experimental data. Note that we focus our model and experimental efforts on the nucleophilic oxidation of methionine because of peroxides in the pH range 2–8 where the oxidation is primarily through water-mediated mechanism.[27]

MATERIALS AND METHODS

Simulation Method

Structure Refinement

In order to generate reliable and accurate starting structures for computational analyses, the X-ray structures obtained from the PDB (Protein Data Bank www.rcsb.org) and models that were generated via homology modeling (see below) were subjected to a protein preparation workflow within the Schrodinger Software MAESTRO (version 9.3), New York, NY. The protocol carries out several steps preprocessing the initial structure identifying missing atoms (especially hydrogen atoms) and residues. The missing residues and atoms are then added using PRIME, a protein modeling tool within the Schrodinger Suite. This step also enumerates bond orders and removes cocrystallized waters. The protocol determines optimal protonation states for histidine residues and then corrects for transposed atoms from arginine, glutamine, and histidine side chains. The next step is optimization of the protein hydrogen bonding network using a cluster-based methodology. The final preparation step is restrained minimization that allows for hydrogen atoms to be freely minimized while heavy atoms have sufficient but limited movement (because of restraints). This final procedure removes atom clashes and is used to relax strained bonds and bond angles.

Homology Modeling

The structures of antibodies (therapeutic1, therapeutic2, and therapeutic3) were obtained through homology modeling as their X-ray structures were not available. Homology modeling was performed using multiple protocols within the Accelrys software DiscoveryStudio (version 3.5), San Diego, CA. The procedure begins with identification of suitable antibody framework templates derived from a database of structurally diverse and comprehensive antibody structures. Using the identified templates, the next step initiates the building of the antibody framework for the conserved "scaffold" regions of the antibody (regions excluding the antibody CDRs). The final part of model building is to generate conformations for the antibody loops. For heavy chain CDR1, CDR2 and light chain CDR1, CDR2 and CDR3 templates are used from the knowledge base of antibody structures that are available (which have already been solved). Heavy chain CDR3 required additional conformation sampling beyond the initial modeling using scaffold templates. The completed models were then processed by the structure refinement protocols described above.

Oxidation Modeling

A molecular dynamics protocol was developed to sample conformations for methionine residues in each of the protein structures. Using DESMOND (Schrodinger LLC, New York, NY) explicit solvent simulations with periodic boundary conditions was set up using an orthorhombic box with SPC waters and the OPLS-2005 force field for each protein. The system was initially relaxed with restraints on solute allowing waters to freely equilibrate, followed by extensive simulation of the entire system without any restraints. The simulation was carried out for 5 ns with a 1-ps recording interval at 300K using a standard protocol. Following the simulation, the 2-shell waters was calculated as the number of water molecules within a radius "*R*" from the sulfur atom in the methionine residue. Chu et al.[27] used $R = 5.5$ Å to account for the size of two water coordination shells. We varied "R" more broadly from 4 to 7 Å to study the effect of radius on the model results. In addition, the solvent-accessible surface area around the methionine side chain as well as the solvent-accessible surface area around the sulfur atom of the methionines (Sulfur–SAA) was calculated at each recorded time point and the statistical average is reported. These SAA were calculated according to the standard procedure with a water probe radius of 1.4 Å.[24]

Experimental Method

All the reagents were purchased from Sigma–Aldrich, St. Louis, MO, unless specified differently. Solution of 1M H_2O_2 was freshly prepared by diluting the stock solution of H_2O_2 (9.79 M) with deionized water and kept on ice prior to use. For forced oxidation experiments, the therapeutic candidates were diluted to