Thiol–Disulfide Exchange in Peptides Derived from Human Growth Hormone

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ABSTRACT: Disulfide bonds stabilize proteins by cross-linking distant regions into a compact three-dimensional structure. They can also participate in hydrolytic and oxidative pathways to form nonnative disulfide bonds and other reactive species. Such covalent modifications can contribute to protein aggregation. Here, we present experimental data for the mechanism of thiol–disulfide exchange in tryptic peptides derived from human growth hormone in aqueous solution. Reaction kinetics was monitored to investigate the effect of pH (6.0–10.0), temperature (4–50°C), oxidation suppressants [ethylenediaminetetraacetic acid (EDTA) and N_2 sparging], and peptide secondary structure (amide cyclized vs. open form). The concentrations of free thiol containing peptides, scrambled disulfides, and native disulfide-linked peptides generated via thiol–disulfide exchange and oxidation reactions were determined using reverse-phase HPLC and liquid chromatography–mass spectrometry. Concentration versus time data were fitted to a mathematical model using nonlinear least squares regression analysis. At all pH values, the model was able to fit the data with $R^2 \geq 0.95$. Excluding oxidation suppressants (EDTA and N_2 sparging) resulted in an increase in the formation of scrambled disulfides via oxidative pathways but did not influence the intrinsic rate of thiol–disulfide exchange. In addition, peptide secondary structure was found to influence the rate of thiol–disulfide exchange. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:1032–1042, 2014

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INTRODUCTION

The rate of failure of candidate drug molecules to successfully obtain regulatory approval is significant, with only one in 10 biopharmaceuticals reaching the market between 2003 and 2010.[1] The successful development of protein drugs has been impeded by physical and chemical instabilities that can result in protein aggregation. Aggregates, in turn, have been associated with altered potency and an increased potential for life-threatening immunogenic side effects.[2–5] The formation of intermolecular disulfide bonds is a common route to the covalent aggregation of therapeutic proteins and other biologics.[6,7] Disulfide bonds are an important component of many therapeutic proteins, including antibodies, enzymes, and hormones, and serve to stabilize the three-dimensional structure by linking distant regions and introducing constraints that maintain the native fold.[8,9] In some cases, disulfide bonds also participate in enzyme catalysis,[10] in the regulation of biological activity,[9] in stabilizing the structure of extracellular proteins,[11] and in protection against oxidative damage.[8] In therapeutic proteins, correct disulfide linkages are critical to the biological activity and stability of this growing class of drugs.[12] For example, nonnative disulfide bonding patterns in IgG antibodies have been associated with changes in receptor binding affinity, stability, and circulating half-life.[13] A disulfide-linked homodimer of human growth hormone (hGH) showed reduced receptor binding affinity and attenuated cell proliferative activity.[14] Human albumin or albumin fusion proteins, which contain 17 disulfides and may contain a free thiol, also may be prone to deleterious disulfide-mediated events.[15] Thus, understanding the mechanisms by which disulfide bonds are disrupted is central to the development of safe and effective protein drug products.

Disulfide bonds can be damaged by a number of chemical reactions, including alkaline hydrolysis via direct attack, α- and β-elimination,[6] free radical attack on one of the sulfur atoms,[16] tryptophan induced reduction of the disulfide bond upon photoexcitation,[17] and, less commonly, via acid–base-assisted hydrolysis.[9] Posttranslational modification of protein disulfide bonds can result in the formation of trisulfides (R-S-S-S-R) by insertion of a sulfur atom as reported for hGH and IgG, albeit without significant effect on activity.[18] Our interest here is one of the most common reactions involving disulfide bonds: thiol–disulfide exchange ($R'SSR'' + RSH \rightarrow R'SSR + R''SH$). In solution, thiol–disulfide exchange occurs when a disulfide ($R'SSR''$) reacts with a dissimilar thiol (RSH), generating the mixed disulfide ($R'SSR$) with the expulsion of the thiol group with the lower pK_a. The mechanism involves S_N2 nucleophilic displacement with the thiolate anion serving as the reactive species,[8,19,20] and with nucleophilic attack of the thiolate anion on the disulfide as the rate determining step.[12] A related reaction, disulfide scrambling ($RSSR + R'SSR' \rightarrow 2R'SSR$) proceeds similarly, but requires initial generation of the thiolate from a disulfide bond. Disulfide scrambling can occur via reduction of the disulfide to regenerate the reactive thiolate anion[8] or a sulfenium cation (RS^+),[21,22] which can then initiate disulfide exchange. Both thiol–disulfide exchange and disulfide scrambling can occur through oxidative pathways as well, in which thiyl radicals ($R'S\bullet$),[23,24] sulfenic acid (RSOH),[25] disulfide radical anion ($R'SSR\bullet^-$),[23] or thiosulfonate ($RS(O)SR'$)[26] groups are the

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reactive species. Thiyl radicals can form disulfide bonds with other thiyl radicals (RSSR) or R′SSR•$^-$ with a thiolate anion.

The rate of thiol–disulfide exchange has been shown to be influenced by factors intrinsic to the protein, including its primary sequence and higher order structure, as well as by the extrinsic properties of the surrounding medium. In particular, primary sequence affects the Cys side chain pK_a[27] and its reactivity. For example, in studies of a model redox-sensitive yellow fluorescent protein,[28] introducing positively charged amino acids adjacent to Cys increased the rate of thiol–disulfide exchange 13-fold by decreasing thiol pK_a and promoting electrostatic interactions with negatively charged glutathione disulfide (GSSG). In studies with cyanogen bromide fragments of hen egg white lysozyme, the rate constants for thiol–disulfide exchange increased 6.5-fold for a Cys with two positively charged neighbors in a 20 mM ionic strength medium relative to a lower ionic strength medium,[29] whereas at high ionic strength, the rate decreased 700-fold. High ionic strength ($\geq$20 mM) was shown to minimize ionic influences from distant residues, thus reducing rate constants for thiol–disulfide exchange. Geometric strain imposed on the disulfide bond by protein native state was shown to affect disulfide reactivity toward dithiothreitol (DTT) in α-lactalbumin.[30] Zhang and Snyder[31] have shown that disulfide bonds in peptides with the sequence Cys-X-Cys, where X is any amino acid, can be reduced easily and peptides containing 4–5 residues between terminal Cys formed very stable loops. Further, addition of Ala C-terminal to Cys did not affect the equilibrium constant for ring closure in either dipeptide (–Cys–Cys–) or tripeptide (–Cys–Val–Cys–) models. Extrinsic factors, particularly those that promote formation of the thiolate anion, also increase the rate of thiol–disulfide exchange. In general, the reaction is accelerated under neutral to alkaline conditions.[32] However, Ryle and Sanger[33] have shown that the reaction occurs even in strongly acidic media (7–12 N HCl) that favor sulfenium ion-mediated exchange. In moderately acidic solutions, no reaction is observed. The effects of temperature on thiol–disulfide exchange reactions have been studied in small organic molecules and proteins. In solution, the reaction follows an Arrhenius relationship with activation energies in the range of 30–70 kJ/mol.[34] In recent studies using single molecule techniques, mechanical force >100 pN promoted thiol–disulfide exchange in the IgG-like fold of cardiac titin domain (I27).[34] This suggests that rates may be affected by fluid shear or other mechanical effects, particularly in surface adsorbed proteins.

While these and other published reports have helped to define the fundamental mechanisms of thiol–disulfide exchange, controlling the reaction in therapeutic proteins remains challenging. For example, it is often difficult to predict the most labile disulfide bonds based on structure alone,[35] suggesting that the structural determinants of thiol–disulfide exchange may be weaker than for other reactions such as deamidation.[36,37] An intact IgG antibody can have more than 20 disulfide bonds, with the potential to form more than 200 species with a single scrambled intra- or intermolecular disulfide. Although "reducible" aggregates are common in protein drug formulations, the disulfide bonds involved are not often identified. There have been reports from our group[38] and others[34] that thiol–disulfide exchange is sensitive to process-induced stresses such as freezing and fluid shear, but the mechanisms of these effects and their interplay with protein structure and fluid composition have not been fully elucidated. As the number of antibodies and antibody- or albumin-fusion drug products increases, the need to control disulfide reactivity becomes more acute.

The long-term goal of this work is to improve the "resolution" of aggregation risk assessment to account for disulfide scrambling by elucidating the mechanisms of thiol–disulfide exchange in pharmaceutically relevant systems (solution and amorphous solids). Proteins are complex macromolecules with multiple reactive centers that can participate in various degradation pathways. Physical and chemical instabilities may be interrelated (e.g., a chemical modification can lead to an unfolding event and vice versa), with both modifications contributing to the overall decomposition rate, though shelf-life is often determined by the rate of formation of individual products. Using model peptides allows the mechanism and rate constant(s) for a specific chemical modification to be determined in the absence of higher order structure and other degradation pathways. This information is a prerequisite for understanding how protein sequence and structure modulate these reactions in intact proteins, and may facilitate better molecular and formulation design approaches. Further, mechanistic information obtained in solution is useful for understanding how nonaqueous environments such as lyophilized solids modulate degradation reactions. Here, we report the effects of pH, temperature, and peptide secondary structure on thiol–disulfide exchange in aqueous solution using peptides derived from hGH.

Human growth hormone is a four-helix bundle protein that belongs to the cytokine family and is used to treat growth disorders and hormone deficiency. With 191 amino acids and two disulfide bonds (no free Cys residues), hGH is a tractable model system for detailed mechanistic studies. hGH is known to aggregate in solution[39,40] and in the solid state,[41–43] facilitated by the formation of scrambled disulfides[14,44,45] and/or the exposure of hydrophobic surfaces.[39,46] In these studies, tryptic peptides derived from the solvent exposed disulfide bond (T20, T20–T21, and cT20–T21, see Table 1) in hGH were used as model compounds to elucidate the mechanism of thiol–disulfide exchange (Scheme 1). The T20 peptide contains Cys182, which has been identified as the most reactive thiol in hGH.[47] T20 was used in its reduced form to react with linear (T20–T21) and cyclic (cT20–T21) peptide models of the native disulfide bond. The results show that for these hGH tryptic peptides, the mechanism of thiol–disulfide exchange is pH independent and the reactions follow Arrhenius behavior. However, the observed rate constant (k_{obs}) depends on the concentration of thiolate anion and hence the solution pH. Additionally, cyclization of the peptide is shown to influence the kinetics of thiol–disulfide exchange.

MATERIALS AND METHODS

Materials

Model peptides T20, T21, T20–T21, and cT20–T21 (see Table 1 for structures) were purchased from GenScript (Piscataway, New Jersey) with >95% purity and supplied as a lyophilized powder. HPLC grade acetonitrile (ACN), NaCl, and KCl were purchased from Fisher Scientific Company (Pittsburgh, Pennsylvania). H_2O_2 and Na_2CO_3 (anhydrous granules) were obtained from Mallinckrodt Baker Inc. (Phillipsburg, New Jersey). K_2HPO_4 and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Company (St. Louis, Missouri). Trifluoroacetic acid (TFA) and formic acid were obtained from Thermo Scientific (Rockford, Illinois). Double-distilled