Thiol–Disulfide Exchange in Peptides Derived from Human Growth Hormone During Lyophilization and Storage in the Solid State

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ABSTRACT: Lyophilization (freeze-drying) is frequently used to stabilize protein therapeutics. However, covalent modifications such as thiol–disulfide exchange and disulfide scrambling can occur even in the solid state. The effects of lyophilization and storage of lyophilized powders on the mechanism and kinetics of thiol–disulfide exchange have not been elucidated and are explored here. Reaction kinetics was monitored in peptides corresponding to tryptic fragments of human growth hormone (T20 + T20–T21 or T20 + cT20–T21) during different stages of lyophilization and during storage of the lyophilized powders at 22°C and ambient RH. The concentrations of reactants and products were determined using RP-HPLC and product identity confirmed using liquid chromatography–mass spectrometry. Loss of native disulfide was observed for the reaction of T20 with both linear (T20–T21) and cyclic (cT20–T21) peptides during the primary drying step; however, the native disulfides were regenerated during secondary drying with no further change till the end of lyophilization. Deviations from Arrhenius parameters predicted from solution studies and the absence of buffer effects during lyophilization suggest that factors such as temperature, initial peptide concentration, buffer type, and concentration do not influence thiol–disulfide exchange during lyophilization. Results from a "cold finger" method used to study peptide adsorption to ice indicate that there is no preferential adsorption to the ice surface and that its presence may not influence disulfide reactivity during primary drying. Overall, reaction rates and product distribution differ for the reaction of T20 with T20–T21 or cT20–T21 in the solid state and aqueous solution, whereas the mechanism of thiol–disulfide remains unchanged. Increased reactivity of the cyclic peptide in the solid state suggests that peptide cyclization does not offer protection against lyophilization and that damage induced by a process stress further affects storage stability at 22°C and ambient RH. J Pharm Sci 104:1291–1302, 2015
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INTRODUCTION

Protein therapeutics continues to grow in commercial and therapeutic importance, providing new treatments for cancer, cardiovascular, and autoimmune diseases. The biologics sector in the United States grew by 18.2% between 2012 and 2013, with sales of $63.6 billion in 2012.[1] Nevertheless, the development of therapeutic proteins can be compromised by the inherent complexity and instability of these macromolecules.[2,3] To improve stability and retain potency, protein pharmaceuticals are often lyophilized.[4–6] Lyophilization (freeze-drying) produces solid powders with high surface area and is used for storage of the protein following expression and for final marketed drug product.[7] Though lyophilization often reduces the rates of chemical and physical degradation, these processes can still occur during manufacturing and subsequent storage in the solid state.[8–10]

Lyophilization cycles typically consist of freezing, primary drying, and secondary drying steps. The process can expose proteins to undesirable stresses such as cold denaturation, increased concentration of solutes and protein ("freeze concentration"), pH changes, and dehydration, all of which can induce protein unfolding and/or structural perturbations.[13,14] Costantino et al.[15] observed secondary structure changes, a decrease in α-helicity and an increase in β-sheet and unordered structure upon lyophilization of human growth hormone (hGH). Lyophilization-induced structural changes have also been reported for recombinant human albumin (rHA).[16] Such structural and/or conformational changes can further lead to aggregation during storage[17] and rehydration.[18,19] Solid-phase aggregation of proteins can occur via a number of mechanisms in the presence of moisture, including thiol–disulfide exchange, disulfide scrambling, nondisulfide covalent aggregation, and noncovalent aggregation.[20] Although there are reports of disulfide-mediated aggregation in the solid state for proteins that contain cysteines and/or disulfide bonds,[21,22] the lack of a complete understanding of factors that influence reactivity reduces formulation to trial-and-error, informed by experience, in selecting composition and stabilizing excipients. Thus, an improved mechanistic understanding of aggregation-inducing processes such as thiol–disulfide exchange will be beneficial for the rational design of formulations that stabilize proteins during lyophilization and storage.

Disulfide bonds increase protein stability by cross-linking distant regions. Native disulfide bonds scramble via oxidative and hydrolytic pathways to form non-native bonds that can affect protein stability and activity. Two predominant pathways lead to disulfide-mediated covalent aggregation: (1) thiol–disulfide exchange ($RSH + R^1SSR^2 \leftrightarrow R^1SSR + R^2SH$) and (2) disulfide scrambling ($RSSR + R^1SSR^1 \leftrightarrow 2R^1SSR$).[23–25] In solution at neutral to alkaline pH, the thiolate anion (RS^-) is the reactive species that initiates thiol–disulfide exchange. Nucleophilic attack of RS^- on a native disulfide (R^1SSR^2) generates a non-native disulfide (R^1SSR) and a new thiol (R^2S^-) in an S_N2

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nucleophilic displacement reaction.[26,27] Disulfide scrambling, a related reaction, is initiated by disulfide bond cleavage to generate a thiolate that then initiates thiol–disulfide or thiol-catalyzed exchange.[20]

Disulfide-mediated aggregation has been reported in lyophilized bovine serum albumin[21] and β-galactosidase.[22] In rHA, lyophilization produced an increase in both β-sheet content and unordered structural elements resulting in partial protein unfolding, which further facilitated moisture-induced aggregation via thiol–disulfide exchange upon storage.[16] Andya et al.[28] observed disulfide-linked dimers and trimers in recombinant humanized monoclonal antibody (rhuMAb) formulations following lyophilization and storage at 30°C. In the absence of excipients, reversible structural alterations during lyophilization promoted covalent aggregate formation upon storage. Degradation reactions can also occur in the solid state in the absence of process-induced structural changes. For example, in the absence of a stabilizing excipient, a rhuMAb (IgG) aggregated in the solid state, though native secondary structure was retained after spray drying.[29] Reports of disulfide-linked aggregates in lyophilized protein samples[21,22,30] demonstrate the importance of designing processes and formulations that can inhibit disulfide bond degradation.

Human growth hormone is a therapeutic protein used to treat growth hormone deficiency and other growth disorders. It has two disulfide bonds and no free thiols, with 191 amino acids the monomeric form has a molecular weight of 22 kDa. Although hGH has no free cysteines, a free thiol may be generated via alkaline hydrolysis during storage and can further facilitate thiol–disulfide exchange reactions.[24] Structural perturbations in hGH have been reported in the solid state[31] and could further result in the formation of disulfide-linked aggregates during storage. A disulfide-linked dimer of hGH (45 kDa) was found to have diminished receptor-binding affinity and cell-proliferative activity.[32] Thus, given its relatively small size, therapeutic value and tendency to aggregate,[33,34] hGH was chosen as a suitable model to study thiol–disulfide exchange kinetics in the solid state.

Previously, we used model peptides derived from the solvent exposed disulfide bond (Table 1) in hGH to investigate the kinetics and mechanism of thiol–disulfide exchange in aqueous solution.[35] Thiol–disulfide exchange reactions between T20 and T20–T21 or cT20–T21 were monitored to study the effect of pH (6.0–10.0), temperature (4°C–50°C), oxidation suppressants [ethylenediaminetetraacetic acid (EDTA) and N_2 sparging] and peptide secondary structure (cyclized vs. linear form) on reaction kinetics. Concentration versus time data were fitted to second-order models to determine kinetic and Arrhenius parameters. We observed that microscopic rate constants for thiol–disulfide exchange were pH independent, whereas k_{obs} values (observed pseudo-first–order-rate constant for the loss of T20–T21; see Table 1 for structure) were pH dependent. The reactions followed Arrhenius behavior with activation energies (Ea) of 39–60 kJ/mol. Activation parameters were consistent with previous reports of thiol–disulfide exchange reactions[36,37] and an S_N2 nucleophilic displacement mechanism. The observed rate constant (k_{obs}) for the loss of T20–T21 depended on the concentration of thiolate anion ($T20S^-$) and hence on solution pH. Excluding oxidation suppressants (EDTA and N_2 sparging) increased the formation of scrambled disulfides via oxidative pathways but did not influence the intrinsic rate of thiol–disulfide exchange. In addition, peptide secondary structure influenced the rate of thiol–disulfide exchange; cyclic peptide reactivity was 10-fold lower than that of the linear form.

The mechanistic information obtained from aqueous solution studies provides a basis for understanding the effects of lyophilization process stresses and the solid environment on thiol–disulfide exchange. Here, we report thiol–disulfide exchange and disulfide scrambling in hGH-derived peptides during lyophilization and subsequent room temperature storage of the lyophilized powders. The hGH-derived peptides were lyophilized without excipients to determine process effects on the mechanism and kinetics of thiol–disulfide exchange and to draw comparisons to the solution–state studies. Formulation and stabilization approaches for protecting native disulfides from thiol–disulfide exchange during lyophilization and subsequent storage were not explored here. The results demonstrate that the rate of thiol–disulfide exchange is accelerated during primary drying, and that peptide secondary structure does not influence reactivity during lyophilization. Factors such as temperature, initial peptide concentration, buffer type and concentration, and peptide adsorption to ice did not influence thiol–disulfide exchange during primary drying. During storage in lyophilized solids, both the rates and the distribution of products differed for linear and cyclic disulfide-containing peptides when compared with those observed in aqueous solution. Peptide cyclization did not offer protection against thiol–disulfide exchange in the solid state; the observed rate constant (k_{obs}) for the loss of cT20–T21 was 10-fold greater than that in aqueous solution.

MATERIALS AND METHODS

Materials

Model peptides T20, T21, T20–T21, rT20–T21, and cT20–T21 (see Table 1 for structures) were purchased from GenScript (Piscataway, New Jersey) with more than 90% purity as a lyophilized powder. HPLC grade acetonitrile (ACN), NaCl, and KCl were purchased from Fisher Scientific Company (Pittsburgh, Pennsylvania). K_2HPO_4, 5,5-dimethyl-1,3-cyclohexanedione (dimedone), EDTA, and sodium citrate tribasic dihydrate were purchased from Sigma Chemical Company (St. Louis, Missouri). Trifluoroacetic acid and formic acid (FA) were obtained from Thermo Scientific (Rockford, Illinois). Double-distilled water (DDI) used for buffer preparation and as HPLC mobile phase was deionized and purified using a Milli-Q water system, (Millipore Ltd., Billerica, Massachusetts) and filtered with a 0.2-μm filter. Glass vials (2 mL) and stoppers (13 mm gray butyl) for lyophilization were purchased from Wheaton (Millville, New Jersey).

Quantification of Reactants and Products by HPLC

Samples were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC; Agilent 1200 series) with UV detection at 215 nm. Agilent Chemstation software was used for data acquisition and analysis. A ZORBAX Eclipse plus C18, 5 μm (4.6 × 250 mm^2) analytical column (Agilent Technologies, Santa Clara, California) was used with gradient elution and the column temperature maintained at 25°C. The gradient elution method and associated calibration plots were as described in our previous work.[38]