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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 thioldisulfide exchange have not been elucidated and are explored here. Reaction kinetics were 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 LC-MS. 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, while 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.

Keywords

lyophilization; freeze-drying; human growth hormone (hGH); peptide; aggregation; kinetics; freezing; disulfide exchange

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

INTRODUCTION

Protein therapeutics continue to grow in commercial and therapeutic importance, providing new treatments for cancer, cardiovascular and autoimmune diseases. The biologics sector in the US grew by 18.2% between 2012-2013, 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 (11, 12). 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. 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) (15). 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, non-disulfide covalent aggregation and non-covalent aggregation (20). While 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: (i) thioldisulfide exchange ($RSH + R^1SSR^2 \leftrightarrow R^1SSR + R^2SH$) and (ii) 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 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 (BSA) (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. observed disulfide-linked dimers and trimers in recombinant humanized monoclonal antibody (rhuMAb) formulations following lyophilization and storage at 30 °C (28). 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 (hGH) 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. While hGH has no free Cys, 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). Thioldisulfide exchange reactions between T20 and T20-T21 or cT20-T21 were monitored to study the effect of pH (6.0-10.0), temperature (4-50 °C), oxidation suppressants (EDTA and N₂ sparging) and peptide secondary structure (cyclized vs. linear form) on reaction kinetics. Concentration vs. 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 while 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 (E_a) 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 N₂ 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 to 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, NJ) with >90% purity as a lyophilized powder. HPLC grade acetonitrile (ACN), NaCl and KCl were purchased from Fisher Scientific Co. (Pittsburgh, PA). K_2HPO_4 , 5,5-dimethyl-1,3-cyclohexanedione (dimedone), ethylenediaminetetraacetic acid (EDTA) and sodium citrate tribasic dihydrate were purchased from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic acid (TFA) and formic acid (FA) were obtained from Thermo Scientific (Rockford, IL). 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, MA) 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, NJ).

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 $\times$ 250 mm) analytical column (Agilent Technologies, Santa Clara, CA) was used with gradient elution and the column temperature maintained at 25 $^{\circ}\text{C}$. The gradient elution method and associated calibration plots were as described in our previous work (35).

Identification of reactants and products on LC-MS—Samples were analyzed using an ESILC/MS system (1200 series LC, 6520 qTOF; Agilent Technologies, Santa Clara, CA) with a ZORBAX 300SB-C18 column (1.0 $\times$ 50 mm, 3.5 μm) with gradient elution similar to that used in HPLC quantitation. Mobile phase A was 0.1% formic acid in water and mobile phase B was 0.1% formic acid in acetonitrile. A gradient run was initiated with 5% B, which was increased to 50% in 6.10 min, then held at 50% for 1 min followed by an increase to 100% in 0.6 min, decreased to 0% in 0.6 min, increased again to 100% in 0.6 min and finally returned to 5% in 0.6 min. The flow rate was maintained at 50 $\mu\text{L}/\text{min}$ and the

column temperature was not controlled. Data were analyzed using MassHunter software. Reactants and products were identified using mass filters with peptide masses corresponding to the different reaction mechanisms for thiol-disulfide exchange and oxidation reactions.

Thiol-disulfide exchange reactions

All peptides were used as provided by the manufacturer without further purification. Stock solutions were prepared in a 0.1% formic acid solution in DDI. Reaction kinetics were monitored during lyophilization and storage of lyophilized powders at 22 °C. For the reaction, 1500 µL of T20 (1125 µM), 1500 µL of T20-T21/cT20-T21 (112.5 µM) and 750 µL of 50 mM phosphate buffer (PB) were added to a 15 mL BD falcon tube and mixed by pipetting. The final pH of the reaction mixture was adjusted using NaOH or HCl (exact volume to be added was determined from pilot studies) after a 100 µL aliquot was removed and quenched with 10 µL 20% formic acid in DDI (FA) to verify initial concentrations ($t = 0$ min before lyophilization). 200 µL aliquots of the reaction mixture were transferred into vials (on ice) and placed inside the freeze-dryer; shelves were pre-cooled to -40 °C. Frozen samples, collected during the freezing step, were thawed and quenched with 20 µL of 20% FA to prevent disulfide exchange prior to analysis. Lyophilization samples at the end of primary drying and during secondary drying were reconstituted with 200 µL of 0.1% FA; 150 µL of the reconstituted sample was quenched with 15 µL of 20% FA. For storage stability studies, reaction mixtures, solution and lyo samples were prepared as described above. At the end of the lyophilization cycle, vials were capped under a vacuum (not sealed) and stored at 22 °C. Samples were withdrawn in triplicate at each time point during lyophilization and storage. 30 µL and 10 µL of the quenched solution were then used for RP-HPLC and LC-MS analysis, respectively. Dilution factors from total reaction volume, addition of NaOH/HCl for pH adjustment and quench solution were accounted for in determining final peptide concentrations. No measurable changes in concentration were observed in quenched samples within the time scale of the experiment.

Buffer effect—T20 and T20-T21 were also lyophilized with 2.5 mM potassium phosphate and 10 mM sodium citrate buffer at pH 7.0 to investigate the effect of buffer type and concentration on thiol-disulfide exchange. Initial peptide concentrations and total reaction volume were as described above. For 2.5 mM PB, 750 µL of 10 mM buffer stock was used and for 10 mM sodium citrate, 750 µL of 50 mM buffer stock was used. Samples were withdrawn in triplicate, quenched and analyzed as described above.

Lyophilization of peptide samples

Solid samples were prepared by lyophilization in a programmable benchtop VirTis freeze-dryer (SP scientific, Gardiner, NY), using methods routine in our labs (38-40) (Table 2). Briefly, the lyophilization cycle consisted of the following steps; freezing at -40 °C, then drying at -35 °C under vacuum (70 mTorr) for 2 h, -5 °C for 8 h, 5 °C for 8 h, 15 °C for 6 h (100 mTorr) and 25 °C for 10 h (100 mTorr). The same lyophilization cycle was used for all solid-state studies to eliminate processing conditions as a variable and was not optimized. The instrument was operated in manual mode to monitor disulfide exchange during lyophilization so that samples could be removed at the end of each step. Lyophilized samples at the end of the cycle appeared as dried powders and did not form elegant cakes

due to the absence of any bulking agents. The glass transition temperature (T_g) of the lyophilized samples was measured using a DSC Q2000 (TA instruments, New Castle, DE) and moisture content was measured using TGA Q5000 (TA instruments, New Castle, DE) and SGA-100 (VTI Corporation, Hialeah, FL).

Peptide adsorption to ice

The rates of thiol-disulfide exchange in hGH model peptides were affected by lyophilization. The role of adsorption to ice in these effects was assessed using a cold finger. A cold finger condenser (24/40 inner joints, Fisher Scientific) was connected with insulated tubing to a circulating water bath (Thermo Scientific) containing an ethylene glycol/water (1:1) mixture and the temperature set to $-10\text{ }^{\circ}\text{C}$. This method was used previously by Kuiper et. al for purification of antifreeze proteins by adsorption to ice (41). The cold finger was first placed in DDI seeded with ice crystals for 10 minutes to form an ice surface. The cold finger was then placed in a solution of peptides inside a Styrofoam box and the ice surface allowed to grow for 1 hour. A peptide solution was prepared by adding 32 mL of T20 (1250 μM), 32 mL of T20-T21 (125 μM) and 16 mL PB buffer (10 mM, pH 7.0, 0.08 M ionic strength, 0.5 mM EDTA and N_2 sparged). After adsorption for 1 hour, the cold finger was removed from the solution and placed inside an empty beaker. The coolant temperature was maintained at $-10\text{ }^{\circ}\text{C}$ for another 6 hours. 100 μL of the solution and thawed ice surface on the cold finger were quenched with 10 μL of 0.1% FA and 30 μL of the quenched samples were injected onto the RP-HPLC column and analyzed for free thiols, native and scrambled disulfides using the method described above.

Data analysis

For the reaction of T20 with linear T20-T21 in the solid state during storage, the data were consistent with a reaction scheme involving: (i) equilibrium ionization of T20 and T21, (ii) irreversible thiol-disulfide exchange reaction of the ionized thiolate form of T20 (T20-S^-) with T20-T21 and (iii) oxidation of the ionized thiolate forms of T20 and T21 (Scheme S1). The scheme is similar to that reported earlier for the reaction in solution (35), but with an irreversible thiol-disulfide exchange reaction rather than a reversible one. The following equations were used to estimate microscopic rate constants:

$$\text{T20S}^- = \text{T20} / \left\{ 1 + \left(10^{-7} \right) / \left(10^{-pK_a} \right) \right\} \quad (1)$$

$$\text{T21S}^- = \text{T21} / \left\{ 1 + \left(10^{-7} \right) / \left(10^{-pK_a} \right) \right\} \quad (2)$$

$$d[\text{T20} - \text{T21}] / dt = -k_1' [\text{T20S}^-] [\text{T20} - \text{T21}] + k_2' [\text{T20S}^-] [\text{T21S}^-] \quad (3)$$

$$d[\text{T20} - \text{T20}] / dt = k_1' [\text{T20S}^-] [\text{T20} - \text{T21}] \quad (4)$$

$$d[\text{T20S}^-] / dt = -k_1' [\text{T20S}^-] [\text{T20} - \text{T21}] - k_2' [\text{T20S}^-] [\text{T21S}^-] \quad (5)$$

$$d[T21S]/dt = k_1' [T20S^-] [T20 - T21] - k_2' [T20S^-] [T21S^-] \quad (6)$$

In the kinetic model, time is the independent variable, reactant and product concentrations are dependent variables, and the rate constants (k_1' and k_2') are treated as parameters to be determined by non-linear regression. The model returned a value of 8.3 for thiol pK_a when it was defined as a parameter to be determined by regression of pH 7.0 data. Thus, the pK_a values for the ionization of T20 and T21 (K_{a20} , K_{a21}) were fixed at 8.3, consistent with previous reports for cysteine (42, 43) and with our previous model for the solution-state reaction (35). Rate constants k_1' and k_2' are second-order rate constants for thioldisulfide exchange and thiol oxidation, respectively (Scheme 1). Kinetic data were fitted to the model (eqns. 1-6) using non-linear regression (SCIENTIST®, Micromath Research, St. Louis, MO). Pertinent models in SCIENTIST® were selected based on reported Model Selection Criterion (MSC) values; a greater MSC value represents a better model. MSC is independent of the scaling of data points and is similar to the Akaike Information Criterion (AIC), which is considered to be a better measure of model validity than R^2 values for non-linear regression (44). For the reaction of T20 with T20-T21 or cT20-T21 in lyophilized powders during storage at 22 °C, the data were fitted to an equation for first-order irreversible reaction to determine the observed rate constant (k_{obs})

$$A/A_0 = \exp(-k_{obs}t) \quad (7)$$

Here, $A = [T20-T21]$ or $[cT20-T21]$, A_0 is the initial concentration of the respective disulfide and t is time.

RESULTS

The mechanisms of thiol-disulfide exchange reactions between T20 and T20-T21 or cT20-T21 have been elucidated in aqueous solution (35) and provide a basis for the quantitative analysis of thiol-disulfide exchange in the solid state. Here, thiol-disulfide exchange reactions were investigated in tryptic peptides derived from hGH during lyophilization and storage of lyophilized powders. Native disulfides are T20-T21 and cT20-T21 and scrambled disulfides formed as products of thiol-disulfide exchange are T20-T20, T21-T21, SMDs and DMD (amino acid sequence of all peptides are shown in Table 1). Figures and tables designated with an “S” can be found in Supplemental Information available online.

Effect of lyophilization on thiol-disulfide exchange

For the reaction of T20 with T20-T21 (thiol: disulfide = 1.4: 1(molar ratio)), primary drying resulted in a 33% loss of native disulfide (Figure 1a). The subsequent increase in T20-T21 levels during secondary drying (Figure 1a) can be attributed in part to the reversibility of the thiol-disulfide exchange reaction. Despite this increase, a net 9% loss remained at the end of secondary drying (Figure 1a). At a greater initial thiol: disulfide ratio of 10:1 (molar ratio), a 24% decrease in T20-T21 was observed after primary drying (Figure 1b). During secondary drying, T20-T21 was regenerated from the monomers and scrambled disulfides, resulting in an overall decrease in native disulfide of 10% at the end of the lyophilization cycle (Figure 1b). Black dotted lines in Figures 1a and 1b are the predicted concentrations of T20-T21

based on solution-state model predictions, adjusted for the changing temperature profile during lyophilization using Arrhenius parameters (see below). The solution-state model provides reasonable predictions during freezing and primary drying, but does not describe concentrations during secondary drying. A mass balance from RP-HPLC and LC-MS data shows that products other than T20-T20 and T21 were not generated in detectable quantities and that 96 % of the initial peptide mass was accounted for at all time points during lyophilization. The distribution of free thiols (T20 and T21), native disulfide (T20-T21) and scrambled disulfide (T20-T20) for the reaction of T20 with T20-T21 at 10:1 is shown in Figure S1.

For the cyclic peptide, a 29% decrease in native disulfide was observed after primary drying (Figure 1b). Like the linear peptide, cT20-T21 was partially regenerated during secondary drying, resulting in a 17% decrease overall in native disulfide content at the end of lyophilization (Figure 1b). The distribution of free thiols (T20 and rT20-T21), native disulfide (cT20-T21) and scrambled disulfide (T20-T20) is shown in Figure S2. In addition to these species, small amounts of single mixed disulfides (SMD1, SMD2) and double mixed disulfide (DMD) (structures in Table 1) were also detected on LC-MS and RP-HPLC (data not shown), though these could not be analyzed quantitatively due to the lack of synthetic standards. Mass balance from RP-HPLC data obtained during freezing, primary and secondary drying accounts for 90% of the initial mass of cT20-T21 (determined from concentrations of cT20-T21 and rT20-T21). The factors that may influence thiol-disulfide exchange during freeze-drying and the results of studies designed to elucidate their effects are described in greater detail below.

Disulfide bond stability during lyophilization

Lyophilization of hGH-derived peptides showed a difference in the distribution of scrambled disulfides when compared to solution studies. Native disulfides (T20-T21 and cT20-T21) were regenerated during secondary drying. For the cyclic peptide, non-native disulfides (SMDs and DMD, Table 1) were detected in the solid state in addition to T20-T20. While previous studies have shown that lyophilization alters thiol-disulfide product distribution (39), the factors contributing to these differences were not identified. Here, we investigated the effects of temperature, peptide concentration, buffer and peptide adsorption to ice on disulfide bond stability during lyophilization.

Effect of temperature and concentration—Arrhenius parameters determined from thermal stress studies in aqueous solution (thiol: disulfide = 1.4:1) were used to predict reaction kinetics at reactant concentrations and temperatures used in lyophilization (black dotted line in Figures 1a and b). At a higher thiol: disulfide ratio (Figure 1b), Arrhenius predictions agree well with data obtained during freezing and primary drying. However, the solution-state model overestimates the extent of thiol-disulfide exchange during secondary drying and does not account for the increase in native disulfide during the transition from primary drying to secondary drying. Deviations from Arrhenius behavior suggest that factors other than temperature and initial peptide concentrations (e.g., phase change, peptide adsorption to ice, peptide structural changes, changes in reaction mechanism and/or freeze

concentration) influence thiol-disulfide exchange during the freeze-drying process, since the model predictions are based on both the concentrations of the reactants and temperature.

Effect of buffer type and buffer concentration—T20 was lyophilized with T20-T21 at pH 7.0 and 4-fold lower buffer concentration (2.5 mM PB, 0.04 M ionic strength, 0.5 mM EDTA and N₂ sparged). The rate of loss of T20-T21 was similar to that observed during lyophilization with 10 mM PB (Figure 2). Similarly, when T20 was co-lyophilized with T20-T21 in sodium citrate buffer (10 mM, 0.5 mM EDTA and N₂ sparged), the native disulfide concentration decreased during primary drying and then increased during secondary drying (Figure 2). While there are some differences in T20-T21 concentration during primary drying, particularly at a low buffer concentration (2.5 mM PB), the T20-T21 concentration is similar at the end of primary drying and after two hours of secondary drying (-5 °C, 70 mTorr) in all three buffers (Figure 2). These results suggest that buffer concentration and type (phosphate vs. citrate) do not contribute to the observed loss of native disulfide during primary drying.

Lyophilization induced damage to disulfide bonds—To investigate the effect of peptide adsorption to an ice surface on thiol-disulfide exchange, a “cold finger” was used (see methods). The ice surface on the cold finger and the solution after adsorption were analyzed using RP-HPLC (data not shown). Peptide concentrations on the ice after adsorption for 1 hr were; [T20] = 326 μM and [cT20-T21] = 33 μM. The results showed that the ratio of T20 to cT20-T21 on the ice (10:1) after adsorption for 1 hr is similar to the ratio (10:1) in the initial reaction mixture before adsorption. Although cT20-T21 does not adsorb preferentially to the ice surface, thiol-disulfide exchange between T20 and cT20-T21 still occurs on ice (6 hr sample thawed and analyzed on the HPLC).

Primary drying duration—The duration of the primary drying step (-35 °C, 70 mTorr) was increased from 2 to 6 hours to determine the effect of drying time on thiol-disulfide exchange (Figure 3). The concentration of native disulfide decreases in the initial 2 hours of primary drying. After this initial loss, the native disulfide is regenerated with continued drying for another 2 hours. Between 4 and 6 hours, there is no further change in T20-T21. While a longer primary drying step can preserve the native disulfide to some extent, lyophilization induced damage to the disulfide bond still occurs and may influence the storage stability of the resulting solids.

Thiol-disulfide exchange in lyophilized powders during storage

The thiol-disulfide exchange reaction between T20 and T20-T21 or cT20-T21 at pH 7.0 (prelyophilization) in lyophilized powders stored at 22 °C was monitored over 0-7 and 0-12 days for T20-T21 and cT20-T21, respectively. Storage stability studies were truncated after 7 days for T20-T21, since oxidative pathways began to dominate (Figure S3). T_g measured for the lyophilized powder at the end of secondary drying was 20.6 °C, and the moisture content at the end of secondary drying and after storage for 14 days at 22 °C was 1-1.5%.

Reaction of T20 with T20-T21—The change in the concentration of T20-T21 during storage is shown in Figure 4a. Rate constants for the exchange (k_1') and oxidation reaction

(k_2') were estimated by fitting the data to the model of Scheme S1. The model provided a good fit to the data; these model fits are represented by solid lines in Figure 4b. The rate constants for the reaction of T20 with T20-T21 in the solid state are shown in (Table 3). Mass balance from RP-HPLC data at the end of 7 days accounts for 95% of initial mass of peptides. The observed pseudo-first order rate constant (k_{obs}) for the loss of T20-T21 during storage in aqueous solution is of the same order as k_{obs} in the solid state, indicating that the reaction is not slowed appreciably in the lyophilized form.

Reaction of T20 with cT20-T21—During storage of the cyclic peptide cT20-T21 in lyophilized powders containing T20, the loss of the disulfide bond was faster in the solid state than in aqueous solutions stored at the same conditions (Figure 5). The concentration of cT20-T21 in solution increases over time due to oxidation as rT20-T21 is oxidized to cT20-T21, behavior similar to that observed for cT20-T21 in aqueous solution studies (35). Mixed disulfides (SMDs and DMD; structures in Table 1) were detected on both RP-HPLC (Figure S4) and LC-MS (data not shown), consistent with the reaction mechanism in Scheme 1b. No other oxidation products such as sulfenic, sulfinic or sulfonic acid were detected. A mass balance from RP-HPLC data at the end of 12 days accounts for 80% of all peptides present in the storage samples. This suggests that the remaining 20% of the initial mass is present as SMDs or DMD, which are not quantitated but detected on LC-MS and RP-HPLC, or as other undetected species. Figure S5 shows a concentration vs. time plot for T20, rT20-T21, native (cT20-T21) and scrambled disulfide (T20-T20) obtained during storage of cyclic peptide. Unlike T20-T21, the concentration of cT20-T21 does not increase during storage after 7 days (Figure 5). This suggests that lyophilization induced stresses favor thiol-disulfide exchange between T20 and cT20-T21, as temperature and pH did not influence reactivity to as great an extent in solution (35).

Observed rate constants (k_{obs}) for the loss of cT20-T21 during storage in lyophilized powders are shown in Table 3. At a pH 7.0, the k_{obs} value for cT20-T21 in the solid state is ~10-fold greater than its k_{obs} value in aqueous solution, and is comparable to k_{obs} for T20-T21 in lyophilized solids at pH 7.0. This suggests that structural constraints imposed by cyclization slow the thiol-disulfide exchange reaction in solution but not in lyophilized solids.

DISCUSSION

The mechanism of thiol-disulfide exchange is well established in aqueous solution. In neutral to basic solution, the thiolate anion is the reactive nucleophile, attacking a disulfide bond via an S_N2 mechanism (26, 27). Thiol-disulfide exchange is known to occur in the solid state as well (16, 17, 21), though the reaction mechanisms have not been fully elucidated. In the studies reported here, hGH-derived model peptides were lyophilized without excipients to investigate the effect of lyophilization on thiol-disulfide exchange kinetics and mechanisms. The amino acid sequences of the model and their masses are shown in Table 1.

In studies of the solution-state reaction of the linear peptide (T20-T21) with T20, the T20-T20 homodimer and T21 were detected as the initial products, as we have reported

previously (35). At longer time points, the T21-T21 homodimer was also observed. These products are consistent with two thiol-disulfide exchange reactions, in which: (i) T20-T21 initially reacts with T20 to produce the T20-T20 homodimer and T21, and (ii) T20-T21 reacts with T21 produced in the first reaction to produce the T21-T21 homodimer and regenerate T20 (Scheme 1a). Solution-state reaction kinetics were not adequately described by a kinetic scheme based only on these two reactions, however, and oxidative pathways were included. This suggests that even in the presence of oxidation suppressants (EDTA and N₂ sparging), T20 and T21 also undergo oxidation to form T20-T20, T21-T21 and T20-T21 via a sulfenic acid intermediate. Kinetic models including both thiol-disulfide exchange and oxidation steps provided excellent fits to data, both in the presence and in the absence of oxidation suppressants (35).

When the reaction of T20-T21 with T20 was monitored during lyophilization and storage in the solid state, T20-T20 and T21 were again detected as initial products, consistent with reaction 1 in Scheme 1a. Reaction 2 in scheme 1a was not observed in the solid state during storage (in the timeframe that the reaction was monitored) based on model fits to the data in SCIENTIST®. As in solution (35), kinetic profiles suggested that T20 and T21 were oxidized to T20-T20, T21-T21 and T20-T21 on extended storage in the solid state (> 7days at 22 °C; Figure S3). Oxidation products such as cysteine sulfenic, sulfinic and sulfonic acid and thiosulfonates were not detected for samples containing T20-T21 colyophilized with T20. However, when T20-T21 was lyophilized without T20 (see SI for method), sulfenic acid and a dimedone adduct were detected (data not shown). The presence of sulfenic acid as an intermediate suggests that disulfide linked peptides can undergo alkaline hydrolysis when a free thiol (T20) is not present, as reported previously (45, 46). Kinetic analysis further suggests that, during lyophilization, T20-T20 and T21 are generated ($k_1 > k_{-1}$) during primary drying and then partly consumed ($k_1 < k_{-1}$) during the first step of secondary drying (Table 2), after which there is no appreciable change in product distribution. During storage in the lyophilized solid, the product distribution is altered and kinetic analysis suggests that thiol-disulfide exchange is essentially irreversible. Possible physicochemical contributions to these kinetic effects are discussed below.

In solution-state reactions of the cyclic peptide (cT20-T21) with T20, only T20-T20 and rT20-T21 were detected as products (35). Mixed disulfides (SMDs and DMD; Table 1) were not observed under any conditions in aqueous solution. After 6 hours, oxidation of rT20-T21 to cT20-T21 was the dominant pathway even under oxidation-suppressed conditions. In contrast, SMDs, DMD, rT20-T21 and T20-T20 were identified as products of the reaction of cT20-T21 with T20 in the solid state. Oxidation of rT20-T21 to cT20-T21 was not the dominant reaction even after 12 days of storage in lyophilized solids at 22 °C, and the concentration of cT20-T21 did not increase with time (Figures 5 and S5). As with the linear peptide, oxidation products such as cysteine sulfenic, sulfinic and sulfonic acid and thiosulfonates were not detected. While sulfenic acid and dimedone adducts were not detected for cT20-T21 lyophilized without T20, trace amounts of cysteine dehydroalanine were detected (data not shown), again suggestive of alkaline hydrolysis in the absence of a free thiol.

In the reaction of the cyclic peptide cT20-T21 with T20 in the solid state, mixed disulfides (SMDs and DMD) were detected in addition to T20-T20 and rT20-T21 during lyophilization and storage (Figure S4, Scheme 1b). Mixed disulfides were not detected in solution studies (35), suggesting that rates of loss from this pool (k_{-3} , k_4) are rapid relative to rates of formation (k_3 , k_{-4}) so that these species do not accumulate in solution (Scheme 1b). The absence of the double mixed disulfide (DMD) in solution can be attributed to the decreased reactivity of cT20-T21 and hence lower concentrations of T20-T20, which in solution did not accumulate to levels sufficient to drive the reaction towards DMD. In contrast, both SMDs and DMD were detected during the primary drying stage of lyophilization, and the native disulfide was regenerated during secondary drying (Table 2).

The observed mechanism of thiol-disulfide exchange between T20 and cT20-T21 is similar to that reported previously for tocinoic acid (TA (ox)) and glutathione (GSH) during lyophilization, where both SMDs and the DMD were detected (39). TA (ox) is a cyclic peptide with 6 amino acids and terminal Cys residues linked by a disulfide bond (Scheme 1b, Table S1). Though the mechanism remains unchanged here, the relative rates of some thiol-disulfide exchange reactions (scheme 1b) are different for cT20-T21 and TA (ox). TA (ox) was consumed in an irreversible thiol-disulfide exchange reaction at the end of lyophilization (39), while for cT20-T21 most of the native disulfide was regenerated at the end of step 3 (Table 2). The results suggest that although the type of disulfide and thiol containing peptides may play a role in reaction kinetics during lyophilization, the overall mechanism for thiol-disulfide exchange in cyclic peptides can be represented by Scheme 1b.

The loss of native disulfide for both T20-T21 and cT20-T21 during the freezing step may be initiated by freeze-concentration, leading to high local concentrations of the peptide reactants (T20 and T20-T21 or cT20-T21). The loss of disulfide bonds during freezing is consistent with previous studies of tumor growth factor- $\beta 1$, in which increased intra- and intermolecular disulfide bond exchange was observed during freezing and long-term storage at $-70\text{ }^{\circ}\text{C}$ (47). Here, deviations from solution Arrhenius parameters (Figure 1) and the absence of a buffer effect suggest that factors such as temperature, peptide concentration, buffer type and concentration have no effect on thiol-disulfide exchange observed during primary drying. Further, the absence of preferential peptide adsorption to ice using the 'cold finger' method suggests that the ice surface itself does not play a role in thiol-disulfide exchange. Instead, loss of the disulfide bond during primary drying may be due to a reduced activation barrier for thiol-disulfide exchange as the environment becomes less polar, as observed at the active site of ribonucleotide reductase (48).

In the simple peptides studied here, most of the native disulfide in T20-T21 and cT20-T21 is regenerated during secondary drying (Figure 1). In larger proteins with multiple disulfide bonds, such reverse reactions may not regenerate the original disulfide bond, but may instead result in intramolecular disulfide scrambling and/or covalent aggregation, depending on the proximity of the groups involved. The role of thiol group proximity to a disulfide bond was investigated previously for small heat shock protein and glutathione (49). The distance between thiols and disulfides, and their relative mobility, can be affected by freeze-drying induced unfolding, particularly for hGH, which is known to undergo structural perturbations during lyophilization (31). The formation of intermolecular disulfide bonds

can further lead to aggregation during storage and/or rehydration. Here, the reactivity of the native disulfide bonds in T20-T21 and cT20-T21 appears to decrease as drying proceeds (Figure 1 and Steps 4, 5 and 6, Table 2), perhaps the result of reduced mobility of the reactive species in the solid state.

The results suggest that, at neutral to slightly alkaline pH, proteins with free thiols and disulfides can undergo thiol-disulfide exchange during lyophilization and storage in the solid state, which can further lead to the formation of disulfide-linked aggregates. Though peptide cyclization retards disulfide exchange in solution, in the solid state the observed disulfide bond degradation for cT20-T21 is greater than in solution and similar to that of the linear peptide, T20-T21. Thus, increased stability of a disulfide bond conferred by secondary structure in solution may not necessarily translate to increased stability in the solid state. Further, structural constraints may not influence disulfide degradation kinetics during lyophilization and storage in the solid state, especially when free thiols are present on the surface of proteins and are in close proximity to a disulfide bond. Thus, the use of lyophilization alone as a stabilizing strategy may not be sufficient to retard thiol-disulfide exchange and offer protection against chemical degradation during storage. Reasonable formulation strategies include restricting free thiol content before lyophilization, using suitable excipients to stabilize protein structure when free thiols are not present on the surface, excluding O₂ from formulations (if products of oxidative pathways are detected) and formulating proteins at low pH followed by reconstitution at near neutral pH.

CONCLUSIONS

The studies reported here detail the effects of lyophilization and storage of lyophilized powders on the mechanisms and rates of thiol-disulfide exchange. Peptide secondary structure does not influence disulfide (T20-T21 or cT20-T21) reactivity in the solid state when co-lyophilized with a free thiol (T20). Further, lyophilization does not retard thiol-disulfide exchange during storage in the solid state. The results provide insight into the effects of process stresses on disulfide exchange and are valuable to the design of robust lyophilization processes for the development of stable peptide and protein drug products that contain free thiols and/or disulfide bonds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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b)

Figure 1.

Change in native disulfide content during lyophilization ($n=3$, $\pm$ SD) at pH 7.0 (10 mM PB, 0.08 M ionic strength, 0.5 mM EDTA and N_2 sparged); I-freezing, II-primary drying and III-secondary drying. Reaction of T20 with a) T20-T21 (thiol: disulfide = 1.4:1), b) T20-T21 ($\blacklozenge$) and cT20-T21 ($\blacksquare$) at thiol: disulfide = 10:1. Black dashed lines indicate the end of each stage during lyophilization and the black dotted lines represent predicted values from solution Arrhenius parameters (see text).

Figure 2.

Effect of buffer type and buffer concentration on thiol-disulfide exchange during lyophilization; freezing (I), primary drying (II) and secondary drying (III). Plot shows change in T20-T21 concentration ($n=3$, $\pm$ SD), co-lyophilized with T20 in 10 mM potassium phosphate buffer (◆), 2.5 mM potassium phosphate buffer (◇) and 10 mM sodium citrate buffer (●). All buffers contain 0.5 mM EDTA and were sparged with N_2 , pH of reaction mixture was adjusted to 7.0 before lyophilization. Initial concentrations of reactants in solution (before lyophilization): $[T20] = 450 \mu\text{M}$; $[T20-T21] = 45 \mu\text{M}$. On the plot, $t=0$ (min) represents solution concentrations before lyophilization.

Figure 3.

Change in concentration of T20-T21 lyophilized with T20 (thiol: disulfide = 6:1, pH 7.0, 10 mM PB, 0.08 M ionic strength, 0.5 mM EDTA and N₂ sparged) during freezing and primary drying (n=3, +/- SD) (◆). Primary drying time was increased from 2 to 6 hours. On the plot, t=0 (min) represents solution concentrations before lyophilization.

a)

b)

Figure 4.

Reaction of T20 with T20-T21 in lyophilized powders stored at 22 °C (n=3, +/- SD). Initial concentrations of reactants in solution (before lyophilization): [T20] = 450 μM; [T20-T21] = 45 μM. Buffer conditions: pH 7.0, 10 mM phosphate buffer, 0.08 M ionic strength, 0.5 mM EDTA and N₂ sparged. a) Change in T20-T21 concentration (▲) and (△) represents the T20-T21 concentration in solution prior to lyophilization and b) concentrations of T20 (■), T21 (●), T20-T20 (□), T21-T21 (○) and T20-T21 (◆). Solid lines are non-linear regressions based on the model in Scheme 1. Initial time point (t = 0 days) corresponds to a sample reconstituted immediately after lyophilization.

Figure 5.

Change in concentration of cT20-T21 lyophilized with T20 (thiol: disulfide = 10:1, pH 7.0, 10 mM PB, 0.08 M ionic strength, 0.5 mM EDTA and N₂ sparged) during storage (n=3, +/- SD) at 22 °C (■), and in solution (◆) (thiol: disulfide = 10:1, pH 7.0, 10 mM PB, 0.08 M ionic strength, 0.5 mM EDTA and N₂ sparged). Both solid and solution samples were stored at 22 °C in lyo vials, n = 3. Open symbols show the initial concentration of cT20-T21 in solution before lyophilization (□) and before storage as solution at 22 °C (◇). Initial data points (t = 0 days, filled symbols) represent solution sample and lyophilized sample reconstituted immediately after lyophilization.

a)

Reaction scheme	Rate constant ($M^{-1}s^{-1}$)			
	k_1	k_{-1}	k_2	k_{-2}
linear peptide, aqueous solution	+	+	+	+
linear peptide, lyophilization	+	+	nd	nd
linear peptide, storage in the solid-state	+	nd	nd	nd

+ : reaction observed
nd- not detected, based on products observed

b)

Reaction scheme	Rate constant ($M^{-1}s^{-1}$)					
	k_3	k_{-3}	k_4	k_{-4}	k_5	k_{-5}
cyclic peptide, aqueous solution	+	+	+	+	nd	nd
cyclic peptide, lyophilization	+	+	+	+	+	+
cyclic peptide, storage in the solid-state	+	+	+	+	+	+

+ : reaction observed
nd- not detected, based on products observed

Scheme 1.

Reaction schemes for thiol-disulfide exchange in a) T20-T21 and b) cT20-T21.

Table 1

Abbreviations and amino acid sequences of peptides detected in solid-state studies with hGH-derived peptides.

Abbreviation	Description	Designation in general reaction scheme	Amino acid sequence	Theoretical mass	Observed mass
T20	Monomeric peptide, free SH group	R_1 and R_2	NH_2 -IVQCR-OH	617.3319	617.3290
T21	Monomelic peptide, free SH group	R_3	NH_2 -SVEGSCGF-OH	784.3062	784.3067
T20-T20	Homodimer, disulfide-Linked	R_1SSR_2 and R_1SSR_1	NH_2 -IVQCR-OH NH_2 -IVQCR-OH	1232.6482	1232.6247
T21-T21	Homodimer, disulfide-Linked	R_3SSR_3	NH_2 -SVEGSCGF-OH NH_2 -SVEGSCGF-OH	1566.5978	1566.5984
T20-T21	Heterodimer, disulfide-Linked	R_2SSR_3	NH_2 -IVQCR-OH NH_2 -SVEGSCGF-OH	1399.6225	1399.6247
CT20-T21	Cyclic peptide, disulfide linked		NH_2 -IVQCRSVEGSCGF-OH	1381.6118	1381.6201
rT20-T21	Linear peptide, free SH groups		NH_2 -IVQCRSVEGSCGF-OH	1383.6275	1383.6344
Single mixed disulfide (SMD)	linear peptide, disulfide linked to T20	OR	NH_2 -IVQCRSVEGSCGF-OH OR NH_2 -IVQCR-OH	1998.9437	1998.9347

Abbreviation	Description	Designation in general reaction scheme	Amino acid sequence	Theoretical mass	Observed mass
Double mixed disulfide (DMD)	linear peptide, disulfide linked to two T20 peptides			2614.2599	2614.2488

Table 2

Lyophilization cycle used for solid-state studies with hGH-derived tryptic peptides.

Step	1	2	3	4	5	6
Temperature (°C)	−40	−35	−5	5	15	25
Duration (h)	2	2	8	8	6	10
Total time (h)	2	4	12	20	26	36
Vacuum (mTorr)	N/A	70	70	70	100	100

N/A, not applicable

Step 1: Freezing, Step 2: primary drying and Steps 3-6: secondary drying

Table 3

Microscopic and observed rate constants for thiol-disulfide exchange between T20 and T20-T21 in lyophilized solids and aqueous solution.

Parameter	Aqueous solution (22 °C), pH 7.0	Lyophilized solids (22 °C), pH 7.0
k_1' (T20-T21)	n/a	$0.45 \pm 0.04 \text{ M}^{-1} \text{ s}^{-1}$
k_2' (T20-T21)	n/a	$9.57 \pm 1.00 \text{ M}^{-1} \text{ s}^{-1}$
k_{obs} (T20-T21)	$1.00 * 10^{-4} \pm 4.00 * 10^{-6} \text{ s}^{-1}^a$	$1.78 * 10^{-4} \pm 1.84 * 10^{-5} \text{ s}^{-1}$
k_{obs} (cT20-T21)	$1.15 * 10^{-5} \pm 1.00 * 10^{-6} \text{ s}^{-1}^a$	$1.56 * 10^{-4} \pm 1.14 * 10^{-5} \text{ s}^{-1}$

n/a -not applicable, different model was used to determine k_1' and k_2'

^aFrom ref 35