Published in final edited form as:

J Pharm Sci. 2014 April ; 103(4): 1032–1042. doi:10.1002/jps.23906.

Thiol-Disulfide Exchange in Peptides Derived from Human Growth Hormone

Saradha Chandrasekhar¹, Daniel E. Epling^{1,2}, Andreas M. Sophocleous^{1,3}, and Elizabeth M. Topp^{1,4}

¹Department of Industrial and Physical Pharmacy, Purdue University, West Lafayette, IN

²Current address: Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, MI

Abstract

Disulfide bonds stabilize proteins by crosslinking distant regions into a compact three-dimensional structure. They can also participate in hydrolytic and oxidative pathways to form non-native 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 were monitored to investigate the effect of pH (6.0-10.0), temperature (4-50 °C), oxidation suppressants (EDTA and N₂ 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 RP-HPLC and LC-MS. Concentration vs. time data were fitted to a mathematical model using non-linear least squares regression analysis. At all pH values, the model was able to fit the data with R² 0.95. Excluding oxidation suppressants (EDTA and N₂ 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.

Keywords

aggregation; thiol-disulfide exchange; peptides; human growth hormone; oxidation; kinetics

INTRODUCTION

The rate of failure of candidate drug molecules to successfully obtain regulatory approval is significant, with only one in ten 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

⁴To whom correspondence should be addressed: Department of Industrial and Physical Pharmacy, Purdue University, 575 Stadium Mall Drive, Room 124D, West Lafayette, IN 47901-2091, Phone: 765-494-1450, Fax: 765-494-6545, topp@purdue.edu.

³Current address: GlaxoSmithKline, King of Prussia, PA

SUPPORTING INFORMATION

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

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, non-native 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 photo-excitation (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 pKa. 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 (RDS) (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 reactive species. Thiyl radicals can form disulfide bonds with other thiyl radicals ($RSSR$) or $R'SSR\bullet^-$ 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 pKa (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

pKa 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), while at high ionic strength, the rate decreased 700-fold. High ionic strength (> 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 towards DTT in α -lactalbumin (30). Zhang et al. 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 (31). 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 have shown that the reaction occurs even in strongly acidic media (7-12 N HCl) that favor sulfenium ion-mediated exchange (33). 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. While “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 non-aqueous 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 human growth hormone (hGH).

Human growth hormone is a 4-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, NJ) with >95% purity and supplied as a lyophilized powder. HPLC grade acetonitrile (ACN), NaCl and KCl were purchased from Fisher Scientific Co. (Pittsburgh, PA). H_2O_2 and Na_2CO_3 (anhydrous granules) were obtained from Mallinckrodt Baker Inc. (Phillipsburg, NJ). K_2HPO_4 and ethylenediaminetetraacetic acid (EDTA) were purchased from Sigma Chemical Co. (St. Louis, MO). Trifluoroacetic acid (TFA) and formic acid 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.

Preparation of peptide stock solutions and disulfide linked peptides for kinetic studies

All peptides were used as provided by the manufacturer. Stock solutions were prepared in a 0.1% formic acid solution in DDI. The T20-T20 and T21-T21 homodimers (Table 1) were synthesized using H_2O_2 as an oxidizing agent according to a method described by Luo et al.

(48). H₂O₂ in phosphate buffer (pH 8.0, 2 mM) was added to solutions of T20 or T21 (4 mM) monomers in a 1:2 molar ratio (total reaction volume < 1 mL) and allowed to react for 1 day. The reaction was then quenched with a 0.5% formic acid solution (2x reaction volume). Conversion of monomer to homodimer was greater than 98% for both monomers, as determined by both RP-HPLC and LC-MS. T20, T21, T20-T21 and cT20-T21 stock solutions were stable at 4 °C for up to 2 weeks and T20-T20, T21-T21 solutions (pH <3.0 after quenching with a 0.5% formic acid solution) were stable for 4 days at 4 °C. Attempts to produce the T20-T21 heterodimer using a similar method resulted in very low yields, so synthetic T20-T21 was purchased from GenScript. Far-UV (190-260 nm) CD analysis with a J-815 CD spectrometer (JASCO, Easton, MD) was used to assess peptide secondary structure.

Buffer preparation

Phosphate buffers (PB) for pH 6.0-8.0 and carbonate buffers (CB) for pH 9.0-10.0 were prepared at different concentrations (10, 20 and 40 mM) to enable extrapolation of rate constants to zero buffer concentration. The ionic strength of all buffers was adjusted to 0.08 M using KCl for PB and NaCl for CB. To minimize oxidation of free thiols, 0.5 mM EDTA and N₂ were used. Buffers were sparged with N₂ for 30 min and then degassed and filtered with a 0.2 µm (Millipore, MA) filter before use. For reactions without suppression of oxidation, PB (pH 7.0, 10 mM) was used without EDTA and N₂.

Quantification of reactants and products by HPLC

RP-HPLC assays were developed to detect and quantify each of the seven species (T20, T20-T20, T20-T21, T21, T21-T21, cT20-T21, rT20-T21) with baseline resolution (Supporting Information Figure S1). Samples were analyzed using reverse-phase high-performance liquid chromatography (RP-HPLC, Agilent 1200 series). The system was equipped with a UV detector operating at 215 nm for all studies. Agilent Chemstation software was used for data acquisition and analysis. A ZORBAX Eclipse plus C18, 5 µm (4.6 × 250 mm) analytical column (Agilent Technologies, Santa Clara, CA) was used with gradient elution (column temperature was maintained at 25 °C). Solvent A was 0.1% TFA (pH~2.5) in water and Solvent B was 0.1 % TFA in acetonitrile. Elution was performed at 1 mL/min starting with 14% Solvent B, which was increased to 47% in 5.2 min, then held at 47% for 3 min and finally returned to 14% at 8.3 min. Total run time for each sample was 12 min. The retention times for T20, T20-T20, T20-T21, T21 and T21-T21 were 4.7 min, 5.2 min, 6.5 min, 7.2 min and 7.9 min respectively, as confirmed by mass spectrometry (ESI-QTOF-MS, Agilent). For studies with the cyclic peptide, a similar gradient elution was used with a total run time of 13 min. The retention times for T20, T20-T20, cT20-T21 and rT20-T21 were 4.8 min, 5.3 min, 7.3 min and 7.7 min, respectively, as confirmed by MS. Calibration curves were linear in the following concentration ranges: 20 µM –1 mM (T20), 10-500 µM (T20-T20), 5-500 µM (T20-T21), 20-180 µM (T21), 10-200 µM (T21-T21), 5-500 µM (cT20-T21), and 5-500 µM (rT20-T21). Limits of detection (S/N = 2) were 15 µM (T20), 2.5 µM (T20-T20), 1 µM (T20-T21), 5 µM (T21), 5 µM (T21-T21) and 5 µM (cT20-T21 and rT20-T21). No cleavage of disulfide bonds was observed at pH levels above the Cys thiol pK_a, as has been previously reported (49). At the end of each experiment, the total mass of the identified products was >98% for pH 6.0-9.0 and >95% for pH 10.0 of the total

initial mass of the reactants. Based on the limits of detection and linear ranges of the calibration curves, initial reactant concentrations of 350 μM (T20) and 250 μM (T20-T21) were used. The amount of T20-T20 and T20-T21 formed via oxidative pathways was determined by mass balance. For example, in the absence of oxidation, the amount of T20-T20 generated will equal the amount of T20-T21 consumed. Any additional T20-T20 formed is assumed to occur via oxidation.

Determination of reaction order

The initial reaction rates were monitored at room temperature (22 °C) and pH 7.0 (10 mM PB, 0.5 mM EDTA, 0.08 M ionic strength, N_2 sparged) in a 2 mL microcentrifuge tube with a total reaction volume of 1250 μL . Initial volumes of T20 were 500 μL , 500 μL and 250 μL , respectively and initial concentrations were calculated based on final concentrations listed below. To determine the reaction order with respect to T20, a final concentration of T20-T21 of 250 μM was used and kinetics monitored at different initial concentrations of T20 (80, 250, 370 and 630 μM). In the same way, the reaction order with respect to T20-T21 was determined using a fixed initial concentration of T20 (350 μM) and different initial concentrations of T20-T21 (130, 270, 410 and 600 μM).

Thiol-disulfide exchange reactions

Reaction kinetics were monitored at five different pH values (6.0, 7.0, 8.0, 9.0, and 10.0). For the reaction, 500 μL of T20 (875 μM), 500 μL of T20-T21 (625 μM) and 250 μL of buffer (50, 100 and 200 mM) were added to a 2 mL microcentrifuge 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 at each pH) after a 100 μL aliquot was removed and quenched to verify initial concentrations ($t = 0$ min). Samples were withdrawn in triplicate at all time points and quenched with 10 μL of 20% formic acid solution in DDI to prevent further scrambling. 30 μL of this quenched solution was then used for RP-HPLC analysis. 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. Reaction kinetics were also monitored at five different temperatures (in triplicate): 4, 15, 25, 40 and 50 °C at pH 7.0 (PB, 10 mM with 0.5 mM EDTA). Initial reactant concentrations, quench conditions and sample analysis were identical to those in the pH studies. The cyclic peptide cT20-T21 (see Table 1) was reacted with T20 in 10 mM PB and CB (0.08 M ionic strength, 0.5 mM EDTA and N_2 sparged). The reaction was monitored kinetically at 22 °C and at 40 °C at pH 7.0 and 9.0 for 6 hours. Initial reactant concentrations were $[\text{T20}] = 450 \mu\text{M}$ and $[\text{cT20-T21}] = 45 \mu\text{M}$. Quench conditions were the same as in studies with T20-T21 and aliquots were withdrawn in triplicate and analyzed using RP-HPLC.

Data analysis

For the reaction of T20 with linear T20-T21, the data were consistent with a reaction scheme involving: (i) equilibrium ionization of T20 and T21, (ii) reversible thiol-disulfide exchange reactions of the ionized thiolate forms of T20 and T21 (i.e., T20-S^- , T21-S^-) with T20-T21 and (iii) oxidation of the ionized thiolate forms of T20 and T21 (Scheme 1). A system of

equations corresponding to this scheme was used to describe the time-varying concentrations of each species and to estimate values of the microscopic rate constants.

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

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

$$\begin{aligned} d[T20 - T21] / dt = & -K_1 [T20S^{-}] [T20 - T21] \\ & + k_2 [T20 - T20] \\ & - k_3 [T21S^{-1}] [T20 - T21] \\ & + k_4 [T21 - T21] [T20S^{-}] \\ & + k_5 [T20S^{-}]^3 [T21S^{-}] \end{aligned} \quad (3)$$

$$d[T21 - T21] / dt = -k_1 [T20S^{-}] [T20 - T21] - k_2 [T20 - T20] [T21S^{-}] + k_5 [T20S^{-}]^3 [T21S^{-}] \quad (4)$$

$$d[T21 - T21] / dt = k_3 [T21S^{-}] [T20 - T21] - k_4 [T21 - T21] [T20S^{-}] + k_6 [T21S^{-}]^2 \quad (5)$$

$$\begin{aligned} d[T20S^{-}] / dt = & -k_1 [T20S^{-}] [T20 - T21] \\ & + k_2 [T20 - T20] [T21S^{-}] \\ & + k_3 [T21S^{-}] + [T20 - T21] \\ & - k_4 [T21 - T21] [T20S^{-}] \\ & - k_5 [T20S^{-}]^3 [T21S^{-}] \end{aligned} \quad (6)$$

$$\begin{aligned} d[T21S^{-}] / dt = & k_1 [T20S^{-}] [T20 - T21] \\ & - k_2 [T20 - T20] [T21S^{-}] \\ & - k_3 [T21S^{-}] [T20 - T21] \\ & + k_4 [T21 - T21] [T20S^{-}] \\ & - k_5 [T20S^{-}]^3 [T21S^{-}] \\ & - k_6 [T21S^{-}]^2 \end{aligned} \quad (7)$$

In the kinetic model, time is the independent variable, reactant and product concentrations are dependent variables, and the rate constants (k_1 , k_2 , k_3 , k_4 , k_5 and k_6) are treated as parameters to be determined by non-linear regression. The model returned a value of 8.3 for thiol pKa when it was defined as a parameter to be determined by regression of pH 7.0 data. Thus, the pKa values for the ionization of the T20 and T21 thiol groups (K_{a20} , K_{a21}) were fixed at 8.3 for all reaction conditions, consistent with previous reports for cysteine (50, 51). Rate constants k_1 , k_2 , k_3 , and k_4 are second-order rate constants for thiol-disulfide exchange; k_5 is a composite rate constant for the production of T20-T21 and T20-T20 by the oxidation

of T20S⁻ and T21S⁻. A composite oxidation rate constant (k_5) was used to minimize model parameters. This composite reaction (R5, Scheme 1) was sufficient to describe the minor oxidation observed when oxidation was suppressed (i.e., with EDTA and N₂ sparging). Kinetic data were fitted to the model (eqns. 1-7) using non-linear regression (SCIENTIST®, Micromath Research, St. Louis, MO). SCIENTIST® reports Model Selection Criterion (MSC) values to determine the most appropriate models; 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 R² values for non-linear models (52). MSC value for the model with composite k_5 was greater than the MSC value for a model with two separate oxidation rate constants. When oxidation was not suppressed, the additional parameter k_6 for the oxidation of ionized T21 to T21-T21 (R6, Scheme 1) was included to compare data to oxidation suppressed studies and to obtain better model fits based on MSC values.

Average values of the measured concentrations of each of the five species (i.e., T20, T21, T20-T20, T21-T21 and T20-T21), as measured by RP-HPLC in triplicate at each time point, defined the data set. In studies of the effect of pH, kinetic studies were repeated at several buffer concentrations and the values of the rate constants extrapolated to zero buffer concentration. An Arrhenius plot was constructed from reactions at various temperatures to estimate the activation energy (E_a) of the reactions. All calculated E_a values were evaluated by one-way ANOVA using SAS, a statistical analysis software (SAS Institute, Cary, NC) at 95% confidence to determine significant differences in E_a among the four thiol-disulfide exchange reactions (R3 and R4, Scheme 1).

For the reaction of T20 with cT20-T21, the data were fitted to the equation for first order irreversible reaction to give k_{obs}

$$A/A_0 = \exp(-k_{obs}t) \quad (8)$$

Here, A = [T20-T21] or [cT20-T21] and A_0 is the initial concentration of the respective disulfide. A reaction scheme was proposed for the reactions with cT20-T21 (Scheme 2).

RESULTS

Addition of oxidation suppressants to favor the thiol-disulfide exchange reaction

Thiol-disulfide exchange reactions were investigated using peptides derived from hGH. Initial studies of the reaction of T20 with T20-T21 in buffer without EDTA or N₂ sparging (pH 7.0) showed that the reactants and products did not appear to reach equilibrium (Figure 1), suggesting another reaction pathway in addition to thiol-disulfide exchange. In reactions of T20 and T20-T21, only five species (i.e., T20, T21, T20-T20, T20-T21, and T21-T21) were detected at all reaction conditions studied (Supporting Information Figure S1). Similarly, in reactions of T20 and cT20-T21, only four species were detected (i.e., T20, T20-T20, cT20-T21, rT20-T21). No additional oxidation products (e.g., sulfenic, sulfinic, or sulfonic acids) were detected with either RP-HPLC or LC-MS both in the presence and absence of oxidation suppressants, strongly suggesting that the additional reaction pathway

is the formation of T20-T20, T20-T21 and T21-T21 from their respective monomers via oxidation. In order to isolate the thiol-disulfide exchange reaction for further investigation, PB buffers (pH 7.0) were sparged with N₂ for 30 minutes, degassed, and different concentrations of EDTA (0.5 mM, 1 mM and 2 mM) were evaluated for their ability inhibit the oxidation reactions. Because the reactions were unaffected by higher EDTA concentrations (data not shown), 0.5 mM was chosen for all subsequent studies in which oxidation was suppressed. Although the rates of the oxidation reactions were significantly attenuated (see below), oxidation could not be eliminated completely using EDTA and N₂ sparging. Other methods to inhibit oxidation like using a nitrogen controlled atmosphere were not investigated as <10% of T20 and T21 participate in the oxidation pathways in the presence of EDTA and N₂ (based on mass balance from RP-HPLC data).

Even in oxidation suppressed conditions, after 6 hours, oxidation of rT20-T21 to cT20-T21 is the major reaction (Supporting Information Figure S2, concentration of cT20-T21 increases while rT20-T21 decreases beyond 500 min). As the main aim was to study thiol-disulfide exchange, we monitored the reaction for up to 6 hours, a time period within which oxidation of T20 to T20-T20 or rT20-T21 to cT20-T21 does not occur to an appreciable extent (determined from pilot studies that were monitored at later time points, Supporting Information Figure S2). Also, mass balance showed that the amount of T20-T20 formed was equivalent to the amount of cT20-T21 consumed, further supporting thiol-disulfide exchange as the major pathway for disulfide scrambling for $t < 6$ h. A similar sampling schedule was used for studies with cT20-T21 at pH 7.0 and T20 and T20-T21 at pH 7.0 and 9.0.

Apparent order of reaction; reaction of T20 and T20-T21

To estimate the order of the disulfide exchange reaction and guide the development of a proposed reaction scheme (Scheme 1), reactant and product concentrations were monitored in the early stages of the reaction (pH 7.0, 10 mM PB, 0.5 mM EDTA and N₂ sparging) where <5% change in both reactants was observed. These studies were conducted for a range of initial concentrations and the apparent first-order reaction rates estimated. Slopes of log-log plots of the rate of reactant disappearance versus reactant concentration were 0.99 ± 0.01 for T20 and 0.93 ± 0.03 for T20-T21 (Figure 2), indicating that the reaction is first-order with respect to the thiol and disulfide reactants and second-order overall. Kuwajima et al. investigated disulfide bond reduction in α -lactalbumin with DTT (30). The authors reported that the kinetics of disulfide bond reduction is rate-limited by inter-molecular disulfide exchange between α -lactalbumin and DTT and that such a reaction is second-order (30), consistent with our findings using hGH-derived peptides. This report and our observation that thiol-disulfide exchange is a second-order reaction are also consistent with other literature reports (8, 19, 53).

Effect of oxidation suppressants on thiol-disulfide exchange

To determine the effect of oxidation on the rate and mechanism of thiol-disulfide exchange at pH 7.0, the rate constants for the exchange and oxidation reactions were estimated by fitting the experimental data from reactions with and without EDTA and N₂ sparging to the model described in Scheme 1 (Figures 1 and 3). The oxidation suppressed thiol-disulfide

exchange data of T20 with T20-T21 were well described by the model in Scheme 1 without R6 ($R^2 = 0.95$, see below), as T21-T21 was formed only via thiol-disulfide exchange in the presence of oxidation suppressants at all pH 6.0-10.0. In the absence of oxidation suppressants, including a separate reaction (R6), described the data better than combining all oxidation reactions into a composite reaction for all three oxidation reactions in Scheme 1 (R5 and R6). For the oxidation of T21 to form T21-T21 in the absence of oxidation suppressants (R6, Scheme 1), the estimated value of rate constant k_6 was $8.9 \pm 2.6 \text{ M}^{-1}\text{s}^{-1}$. In the presence of oxidation suppressants, oxidation of T21 was not observed ($k_6 = 0$). Rate constants for thiol-disulfide exchange were similar with and without oxidation suppressants, while k_5 increased 3.6-fold and k_6 increased from 0 to $8.9 \pm 2.6 \text{ M}^{-1}\text{s}^{-1}$ (Figure 4). Statistical analysis showed no significant difference in rate constants for thiol-disulfide exchange ($p > 0.05$) and a significant difference for the oxidation reaction rate constant ($p < 0.05$) in the presence and absence of EDTA and N_2 , indicating that the intrinsic rate of the disulfide exchange reactions are not affected by the presence of oxidation side-reactions.

Effect of pH on the reaction of T20 with T20-T21

Thiol-disulfide exchange reactions were monitored as a function of buffer concentration to determine the effect of buffer catalysis. At pH 6.0, 8.0, 9.0 and 10.0, a high buffer concentration (40 mM) appears to have had a moderate stabilizing effect (Supporting Information Figure S3). Statistical analysis showed no significant difference between extrapolated rate constants and the rate constants at 10 mM buffer ($p > 0.05$). Nevertheless, at all pH values, we report rate constants extrapolated to zero buffer concentration. An example of the fit of the data to the model (Scheme 1) at pH 7.0 is shown in Figure 3; similar fits were obtained at the other pH values (Supporting Information Figure S4). At pH 7.0, the extrapolated rate constants of the reactions shown in Scheme 1 were: $k_1 = 5.0 \pm 0.2 \text{ M}^{-1}\text{s}^{-1}$, $k_2 = 10.6 \pm 0.3 \text{ M}^{-1}\text{s}^{-1}$, $k_3 = 3.4 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$, $k_4 = 6.9 \pm 0.1 \text{ M}^{-1}\text{s}^{-1}$. Rate constants at other pH values and buffer concentrations are in Supporting Information Table S1. A pH-rate profile for pH 6.0-10.0 is shown in Figure 5 for the extrapolated rate constants. Statistical analysis showed no significant difference ($p > 0.05$) in the rate constants for this pH range. This suggests that the mechanism of thiol-disulfide exchange is independent of pH as reported by others (54, 55). Although a change in solution pH did not affect the rate of thiol-disulfide exchange in tryptic peptides, it can influence reaction kinetics in proteins, which can undergo pH-induced conformational changes that can alter the exposure of disulfides and/or free thiols. The pseudo-first order rate constant (k_{obs}) for the loss of T20-T21 was determined by monitoring the change in concentration over time. Data at different pH values (6.0-10.0) are shown as a pH-rate profile in Figure 6. The slope of the pH-rate profile in the pharmaceutically relevant range of pH 6.0 to 8.0, below the thiol pKa (8.3), is ~ 1 . The change in k_{obs} with increasing pH (i.e., the slope of the pH rate profile) decreases above pH 8.0. However, there is a slight increase in k_{obs} above pH 8.0 as the population of thiolate anion still changes, albeit at decreasing proportions at $\text{pH} > \text{thiol pKa}$. The observed pH dependence of k_{obs} and the pH independence of k_1 - k_4 indicate that thiol-disulfide exchange depends on the concentration of thiolate anion.

Activation parameters for the reaction of T20 with T20-T21

Microscopic rate constants for thiol-disulfide exchange (k_1 , k_2 , k_3 , and k_4) obtained from model fits to data at 4, 15, 35, 40 and 50 °C were used to construct an Arrhenius plot (Figure 7, see Supporting Information Figure S5 for Arrhenius plot of k_5). The resulting Arrhenius parameters can be used to predict the extent to which temperature changes during storage and processing conditions can affect thiol-disulfide exchange. The thiol-disulfide exchange reaction was found to follow linear Arrhenius behavior in the temperature range 4-50 °C. Linear Arrhenius plots suggest that a change in temperature within this range does not result in a change in the proposed reaction mechanism or the rate-limiting step (Scheme 1).

There is no significant difference ($p > 0.05$) in the activation energy values for any of the thiol-disulfide exchange reactions in Scheme 1 (R3 and R4) based on a one-way ANOVA. R3 and R4 represent two mechanistically equivalent reversible reactions and thus would be expected to have similar activation energies. We estimated Arrhenius activation energies for the reaction of T20 and T20-T21 to be in the range of 41-53 kJ/mol (Table 2). An Eyring plot (Supporting Information Figure S6) was used to estimate values for enthalpy ($\Delta H^\ddagger$), entropy ($\Delta S^\ddagger$) and free energy of activation ($\Delta G^\ddagger$) at 25 °C (Table 2).

Effect of peptide cyclization on thiol-disulfide exchange

The non-cyclic model peptides (T20, T21, T20-T20, T21-T21 and T20-T21) do not have any secondary structure, as confirmed by far-UV CD analysis. However, the CD spectrum for cT20-T21 shows internal hydrogen bonding, possibly due to the presence of a beta-turn-like structure (Supporting Information Figure S7). Initial studies carried out with T20 and cT20-T21 with the same molar ratios as the heterodimer study (thiol: disulfide = 1.4:1) showed no reaction at 22, 40, or 60 °C. Thus, a higher thiol: disulfide ratio of 10:1 was used for kinetic studies using cT20-T21. We compared the rate of reaction of T20 with cT20-T21 at two different pH values and temperatures with that of T20-T21 under similar reaction conditions (Table 3) to determine the effect of peptide secondary structure on reaction kinetics. For scrambling reactions involving cT20-T21 and T20, rates were considerably slower than for the linear form (Figure 8). Even with a 10:1 molar ratio of thiol: disulfide, only a 26% decrease in disulfide content was observed after 6 hours at pH 9.0 and 22 °C, as compared to an 86% decrease for the linear T20-T21. Our results suggest that secondary structure has a marked effect on the reaction rate, and that secondary structural constraints may attenuate thiol-disulfide exchange in intact hGH.

DISCUSSION

At neutral to basic pH, the mechanism of thiol-disulfide exchange involves reaction of the thiolate anion (RS^-) with a disulfide bond ($R'SSR''$) (8, 9, 56). The thiol group undergoes deprotonation to form a reactive nucleophile (RS^-), followed by the exchange of redox equivalents leading to oxidation of the attacking thiol and reduction of $R'SSR''$ (8). In our studies with model peptides derived from hGH, products consistent with this mechanism were detected, including T20-T20, T21, T21-T21 and rT20-T21. No additional oxidation products such as cysteine sulfenic acid, cysteine sulfinic acid, cysteine sulfonic acid or thiosulfonates were detected, either in the presence or absence of EDTA and N_2 , as

confirmed by MS analysis and mass balance upon completion of the kinetic studies. While previous reports of thiol-disulfide exchange have employed low molecular weight thiols such as GSH (19, 56, 57) and Cys-SH (20) as reactants, here we report thiol-disulfide exchange using model peptides (T20-T21, cT20-T21, T20) representative of proteins containing unpaired Cys and disulfide bonds. Although native hGH and most mAbs generally do not have unpaired Cys, these may be generated by incomplete folding or disulfide bond reduction during expression, folding, purification and/or fill-finish operations, and during storage in solution or solid formulations.

The reaction between thiols and disulfides has been studied in detail by other groups (32, 53). A data modeling approach was used previously by Luo et al. to determine oxidation reaction kinetics for the reaction of Cys-SH and H_2O_2 (48) and we have used a similar approach. To our knowledge, however, this is the first report of a detailed kinetic model allowing the determination of both observed and microscopic rate constants for thiol-disulfide exchange in peptide model compounds. In the kinetic model, reactions R1-R5 (Scheme 1) were sufficient to describe both thiol-disulfide exchange and thiol oxidation in studies with oxidation suppressants. Thiol oxidation reactions R5 and R6 represent composite reactions that involve oxidation of T20 or T21 via a sulfenic acid (RSOH) intermediate (48). RSOH is a reactive species that is completely consumed, was not detected analytically and as a result was excluded from the reaction scheme. Excellent fits with $R^2 = 0.95$ suggest that the reactions represented by Scheme 1 effectively describe the mechanism for thiol-disulfide exchange between T20 and T20-T21 and oxidation of free thiols (T20, T21) to form homodimers (T20-T20, T21-T21) and heterodimer (T20-T21). In the reaction of T20 with T20-T21, omitting oxidation suppressants (EDTA and N_2 sparging) resulted in an increase in the oxidation rate constant k_5 by 366% and k_6 to $8.9 \pm 2.6 \text{ M}^{-1} \text{ s}^{-1}$ from ~ 0 , while the other rate constants remained unchanged (Supporting Information Table S1). The k_{obs} value for the loss of T20 decreased by 130% in the presence of oxidation suppressants while k_{obs} for the loss of T20-T21 remained unchanged. The results show that excluding oxidation suppressants accelerates the formation of scrambled disulfides (T20-T20 and T21-T21) via oxidative pathways. Hydroxyl ion mediated disulfide exchange and aggregation has been reported for atrial natriuretic peptide (ANP), a cyclic peptide with 25 amino acids and a disulfide bond (C4-C28) in the solid state (49). However, alkaline hydrolysis of peptide bonds in T20-T21 and cT20-T21 was not observed up to pH 10.0.

Rate constants for the reaction of T20 with T20-T21 are comparable to previous reports. For example, Holmgren et al. studied disulfide bond reduction in insulin with dithiothreitol (DTT) at neutral pH and determined a second-order rate constant for the reaction of $5 \text{ M}^{-1} \text{ s}^{-1}$ (58). In another study with DTT, Wiita et al. studied force-dependent chemical kinetics of disulfide bond reduction (34). In the absence of an external force and at pH 7.2, a second-order rate constant of $6.45 \text{ M}^{-1} \text{ s}^{-1}$ was obtained. These values for thiol mediated disulfide exchange are similar to our model predictions for the reactions of both T20 and T21 with T20-T21 (Scheme 1, R3 and R4) at pH 7.0 ($3\text{--}11 \text{ M}^{-1} \text{ s}^{-1}$).

Arrhenius activation energy values reported here (Table 2) are similar to those obtained by Fernandes et al., who reported E_a values for thiol-disulfide exchange in solution in the range 60–66 kJ/mol (59). Wiita et al. assumed values for the Arrhenius parameter (A) of $10^5\text{--}10^{12}$

$\text{M}^{-1}\text{s}^{-1}$, and obtained activation energies between 30-65 kJ/mol for thiol-disulfide exchange (34). Whitesides et al. determined activation parameters for the reaction of Ellman's reagent and glutathione with three different thiols in water at 30 °C (56); $G^\ddagger = 44.4 - 65.7$ kJ/mol, $H^\ddagger = 37.7 - 66.9$ kJ/mol and $S^\ddagger = -19.7 - +13.4$ J/mol.K. The activation parameters reported here, with the exception of more negative values of $S^\ddagger$, are similar to values cited above. Thiol-disulfide exchange proceeds via a linear $\text{S}_{\text{N}}2$ mechanism (60, 61), with greater translational and vibrational constraint of the transition state than the reactants, resulting in a negative $S^\ddagger$ and a positive $G^\ddagger$. The values of the activation parameters reported here are consistent with expected values of $-85 - -125$ J/mol.K for this mechanism (56) and suggest that translational and vibrational constraints play a more significant role for thiol-disulfide exchange reactions involving peptides than in small molecules. Youngman et al. have shown that carbamidomethylation of all four Cys residues in hGH decreases stability by ~ 37.7 kJ/mol (62). Protein destabilization by disulfide bond reduction increased the population of self-associated forms. This is similar to the activation energies reported here, suggesting that the activation energy for thiol-disulfide exchange in model peptides is of the same order as the decrease in stability that results from the reduction of disulfide bonds in native hGH.

The results also show that the reaction is ten-fold slower in the cyclic peptide (cT20-T21) than in the linear form (T20-T21), suggesting that secondary structural constraints in hGH slow thiol-disulfide exchange. Interestingly, this observation is contrary to previous reports. Rabenstein et al. determined observed rate constants for thiol-disulfide exchange with Cys and Arg-vasopressin (AVP) at pH 7.0 and 25 °C (53). AVP is a cyclic peptide with 9 amino acid residues. The reaction of AVP with Cys (CysSH) was 100 times faster than the reaction between glutathione disulfide (GSSG) and CysSH. The authors attributed this to several factors including a more accessible disulfide bond formed between two terminal Cys residues in AVP. The disulfide bond in cT20-T21 (C4-C11) is somewhat removed from the N- and C-termini, and may be less solvent exposed than in T20-T21, perhaps contributing to the reduction in rate observed here. Internal hydrogen bonding in the cT20-T21 peptide (CD spectrum, Supporting Information Figure S7) could impose a structural constraint which translates to reduced reactivity of the disulfide bond with T20. Additionally, in peptides with 1-3 residues between the Cys, cyclization is unfavorable due to geometric strain, while a stable disulfide bond is formed in peptides with 4-5 intervening amino acids (31). The differences in structural effects between the studies reported here and previous reports indicate that the relationship between peptide secondary structure and reactivity in thiol-disulfide exchange is not completely understood, and that further studies are warranted, particularly given the relevance to therapeutic proteins such as hGH, monoclonal antibodies (mAbs) and human serum albumin.

A "molecular design intent" process to evaluate key quality attributes early in development can improve candidate selection, and may focus on attributes such as target affinity, molecule stability and manufacturing yield. Typically, evaluating a protein drug candidate's risk of short product shelf-life consists of assessing the potential for deamidation, oxidation and aggregation. Although aggregation is a complex phenomenon with various underlying mechanisms, the risks of all potential aggregation pathways are often grouped together because little is known about how to predict the risks individually.

This can result in overlooking or underestimating a relevant aggregation pathway, such as disulfide scrambling, during candidate evaluation. Kinetic and thermodynamic parameters for thiol-disulfide exchange reactions in model peptides from hGH measured in the absence of higher order structure and other degradation pathways are reported here. The results can be extended to therapeutic proteins and biosimilars to determine the influence of sequence, structure and processing conditions on reaction mechanisms and disulfide-mediated aggregation propensity and to improve candidate selection during development.

CONCLUSION

The studies reported here define the mechanistic details of thiol-disulfide exchange and oxidation in hGH model peptides in solution. Microscopic and apparent rate constants obtained from model predictions provide an insight into the effects of pH, temperature and secondary structure on thiol-disulfide exchange. Detailed kinetic analysis of degradation pathways like thiol-disulfide exchange is valuable for the development of protein drug formulations, both in solution and in the solid state. Further, the information can be used to improve our understanding of the contributions of thiol-disulfide exchange to aggregation in other therapeutic proteins with disulfide bonds and/or Cys residues.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENT

The authors gratefully acknowledge financial support from NIH R01 GM085293, Purdue Research Foundation (graduate fellowship, SC) and Purdue University.

REFERENCES

1. Knablein, J.r. Modern biopharmaceuticals : recent success stories. Wiley-Blackwell; Weinheim: 2013.
2. Jiskoot W, Randolph TW, Volkin DB, Middaugh CR, Schoneich C, Winter G, Friess W, Crommelin DJ, Carpenter JF. Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. *J Pharm Sci.* 2012; 101:946–954. [PubMed: 22170395]
3. Schellekens H. Immunogenicity of therapeutic proteins: clinical implications and future prospects. *Clin Ther.* 2002; 24:1720–1740. discussion 1719. [PubMed: 12501870]
4. Schellekens H. The immunogenicity of therapeutic proteins. *Discov Med.* 2010; 9:560–564. [PubMed: 20587346]
5. Wang W, Singh SK, Li N, Toler MR, King KR, Nema S. Immunogenicity of protein aggregates--concerns and realities. *Int J Pharm.* 2012; 431:1–11. [PubMed: 22546296]
6. Trivedi MV, Laurence JS, Siahaan TJ. The role of thiols and disulfides on protein stability. *Curr Protein Pept Sci.* 2009; 10:614–625. [PubMed: 19538140]
7. Wang, W.; Roberts, CJ. Aggregation of therapeutic proteins. Wiley; Hoboken, N.J.: 2010.
8. Gilbert HF. Thiol/disulfide exchange equilibria and disulfide bond stability. *Methods Enzymol.* 1995; 251:8–28. [PubMed: 7651233]
9. Hogg PJ. Disulfide bonds as switches for protein function. *Trends Biochem Sci.* 2003; 28:210–214. [PubMed: 12713905]
10. Holmgren A. Thioredoxin. *Annu Rev Biochem.* 1985; 54:237–271. [PubMed: 3896121]

11. Klink TA, Woycechowsky KJ, Taylor KM, Raines RT. Contribution of disulfide bonds to the conformational stability and catalytic activity of ribonuclease A. *Eur J Biochem.* 2000; 267:566–572. [PubMed: 10632727]
12. Zhang L, Chou CP, Moo-Young M. Disulfide bond formation and its impact on the biological activity and stability of recombinant therapeutic proteins produced by *Escherichia coli* expression system. *Biotechnol Adv.* 2011; 29:923–929. [PubMed: 21824512]
13. Liu H, May K. Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. *MAbs.* 2012; 4:17–23. [PubMed: 22327427]
14. Bustamante JJ, Grigorian AL, Munoz J, Aguilar RM, Trevino LR, Martinez AO, Haro LS. Human growth hormone: 45-kDa isoform with extraordinarily stable interchain disulfide links has attenuated receptor-binding and cell-proliferative activities. *Growth Horm IGF Res.* 2010; 20:298–304. [PubMed: 20472479]
15. Kratz F. Albumin as a drug carrier: Design of prodrugs, drug conjugates and nanoparticles. *Journal of Controlled Release.* 2008; 132:171–183. [PubMed: 18582981]
16. Kice JL. Electrophilic and nucleophilic catalysis of scission of sulfur-sulfur bond. *Accounts Chem Res.* 1968; 1:58.
17. Wu L-Z, Sheng Y-B, Xie J-B, Wang W. Photoexcitation of tryptophan groups induced reduction of disulfide bonds in hen egg white lysozyme. *Journal of Molecular Structure.* 2008; 882:101–106.
18. Gu S, Wen D, Weinreb PH, Sun Y, Zhang L, Foley SF, Kshirsagar R, Evans D, Mi S, Meier W, Pepinsky RB. Characterization of trisulfide modification in antibodies. *Anal Biochem.* 2010; 400:89–98. [PubMed: 20085742]
19. Rabenstein DL, Weaver KH. Kinetics and Equilibria of the Thiol/Disulfide Exchange Reactions of Somatostatin with Glutathione. *J Org Chem.* 1996; 61:7391–7397. [PubMed: 11667665]
20. Rabenstein DL, Yeo PL. Kinetics and Equilibria of the Formation and Reduction of the Disulfide Bonds in Arginine-Vasopressin and Oxytocin by Thiol/Disulfide Interchange with Glutathione and Cysteine. *Journal of Organic Chemistry.* 1994; 59:4223–4229.
21. Benesch RE, Benesch R. The mechanism of disulfide interchange in acid solution - role of sulfenium ions. *Journal of the American Chemical Society.* 1958; 80:1666–1669.
22. Manning MC, Patel K, Borchardt RT. Stability of protein pharmaceuticals. *Pharm Res.* 1989; 6:903–918. [PubMed: 2687836]
23. Carballal S, Alvarez B, Turell L, Botti H, Freeman BA, Radi R. Sulfenic acid in human serum albumin. *Amino Acids.* 2007; 32:543–551. [PubMed: 17061035]
24. Mozziconacci O, Kerwin BA, Schoneich C. Photolysis of an intrachain peptide disulfide bond: primary and secondary processes, formation of H₂S, and hydrogen transfer reactions. *J Phys Chem B.* 2010; 114:3668–3688. [PubMed: 20178349]
25. Regazzoni L, Panusa A, Yeum KJ, Carini M, Aldini G. Hemoglobin glutathionylation can occur through cysteine sulfenic acid intermediate: electrospray ionization LTQ-Orbitrap hybrid mass spectrometry studies. *J Chromatogr B Analyt Technol Biomed Life Sci.* 2009; 877:3456–3461.
26. Giles GI, Jacob C. Reactive sulfur species: an emerging concept in oxidative stress. *Biol Chem.* 2002; 383:375–388. [PubMed: 12033429]
27. Summa D, Spiga O, Bernini A, Venditti V, Priora R, Frosali S, Margaritis A, Di Giuseppe D, Niccolai N, Di Simplicio P. Protein-thiol substitution or protein dethiolation by thiol/disulfide exchange reactions: the albumin model. *Proteins.* 2007; 69:369–378. [PubMed: 17607746]
28. Hansen RE, Ostergaard H, Winther JR. Increasing the reactivity of an artificial dithiol-disulfide pair through modification of the electrostatic milieu. *Biochemistry.* 2005; 44:5899–5906. [PubMed: 15823049]
29. Snyder GH, Cennerazzo MJ, Karalis AJ, Field D. Electrostatic influence of local cysteine environments on disulfide exchange kinetics. *Biochemistry.* 1981; 20:6509–6519. [PubMed: 6796114]
30. Kuwajima K, Ikeguchi M, Sugawara T, Hiraoka Y, Sugai S. Kinetics of disulfide bond reduction in alpha-lactalbumin by dithiothreitol and molecular basis of superreactivity of the Cys6-Cys120 disulfide bond. *Biochemistry.* 1990; 29:8240–8249. [PubMed: 2123714]

31. Zhang RM, Snyder GH. Dependence of formation of small disulfide loops in two-cysteine peptides on the number and types of intervening amino acids. *J Biol Chem.* 1989; 264:18472–18479. [PubMed: 2808384]
32. Szajewski RP, Whitesides GM. Rate constants and equilibrium-constants for thiol-disulfide interchange reactions involving oxidized glutathione. *Journal of the American Chemical Society.* 1980; 102:2011–2026.
33. Ryle AP, Sanger F. Disulphide interchange reactions. *Biochem J.* 1955; 60:535–540. [PubMed: 13249946]
34. Wiita AP, Ainarapu SR, Huang HH, Fernandez JM. Force-dependent chemical kinetics of disulfide bond reduction observed with single-molecule techniques. *Proc Natl Acad Sci U S A.* 2006; 103:7222–7227. [PubMed: 16645035]
35. Shaked Z, Szajewski RP, Whitesides GM. Rates of thiol-disulfide interchange reactions involving proteins and kinetic measurements of thiol pKa values. *Biochemistry.* 1980; 19:4156–4166. [PubMed: 6251863]
36. Xie M, Schowen RL. Secondary structure and protein deamidation. *J Pharm Sci.* 1999; 88:8–13. [PubMed: 9874696]
37. Robinson NE, Robinson AB. Molecular clocks. *Proc Natl Acad Sci U S A.* 2001; 98:944–949. [PubMed: 11158575]
38. Thing M, Zhang J, Laurence J, Topp EM. Thiol-disulfide interchange in the tocinoic acid/ glutathione system during freezing and drying. *J Pharm Sci.* 2010; 99:4849–4856. [PubMed: 20821392]
39. Otzen DE, Knudsen BR, Aachmann F, Larsen KL, Wimmer R. Structural basis for cyclodextrins' suppression of human growth hormone aggregation. *Protein Sci.* 2002; 11:1779–1787. [PubMed: 12070330]
40. St John RJ, Carpenter JF, Randolph TW. High pressure fosters protein refolding from aggregates at high concentrations. *Proc Natl Acad Sci U S A.* 1999; 96:13029–13033. [PubMed: 10557267]
41. Pikal MJ, Dellerman KM, Roy ML, Riggan RM. The effects of formulation variables on the stability of freeze-dried human growth hormone. *Pharm Res.* 1991; 8:427–436. [PubMed: 1871037]
42. Pikal MJ, Rigsbee D, Roy ML, Galreath D, Kovach KJ, Wang B, Carpenter JF, Cicerone MT. Solid state chemistry of proteins: II. The correlation of storage stability of freeze-dried human growth hormone (hGH) with structure and dynamics in the glassy solid. *J Pharm Sci.* 2008; 97:5106–5121. [PubMed: 18351639]
43. Pikal MJ, Rigsbee DR, Roy ML. Solid state chemistry of proteins: I. glass transition behavior in freeze dried disaccharide formulations of human growth hormone (hGH). *J Pharm Sci.* 2007; 96:2765–2776. [PubMed: 17621677]
44. Lewis UJ, Peterson SM, Bonewald LF, Seavey BK, VanderLaan WP. An interchain disulfide dimer of human growth hormone. *J Biol Chem.* 1977; 252:3697–3702. [PubMed: 863899]
45. Junnila RK, Wu Z, Strasburger CJ. The role of human growth hormone's C-terminal disulfide bridge. *Growth Horm IGF Res.* 2013; 23:62–67. [PubMed: 23478141]
46. St John RJ, Carpenter JF, Balny C, Randolph TW. High pressure refolding of recombinant human growth hormone from insoluble aggregates. Structural transformations, kinetic barriers, and energetics. *J Biol Chem.* 2001; 276:46856–46863. [PubMed: 11591710]
47. Wang, YJ.; Pearlman, R. Stability and characterization of protein and peptide drugs : case histories. Plenum Press; 1993.
48. Luo D, Smith SW, Anderson BD. Kinetics and mechanism of the reaction of cysteine and hydrogen peroxide in aqueous solution. *J Pharm Sci.* 2005; 94:304–316. [PubMed: 15570599]
49. Wu SL, Leung D, Tretyakov L, Hu J, Guzzetta A, Wang YJ. The formation and mechanism of multimerization in a freeze-dried peptide. *Int J Pharm.* 2000; 200:1–16. [PubMed: 10845681]
50. Krekel F, Samland AK, Macheroux P, Amrhein N, Evans JN. Determination of the pKa value of C115 in MurA (UDP-N-acetylglucosamine enolpyruvyltransferase) from *Enterobacter cloacae*. *Biochemistry.* 2000; 39:12671–12677. [PubMed: 11027147]
51. Tajc SG, Tolbert BS, Basavappa R, Miller BL. Direct determination of thiol pKa by isothermal titration microcalorimetry. *J Am Chem Soc.* 2004; 126:10508–10509. [PubMed: 15327286]

52. Spiess AN, Neumeyer N. An evaluation of R2 as an inadequate measure for nonlinear models in pharmacological and biochemical research: a Monte Carlo approach. *BMC Pharmacol.* 2010; 10:6. [PubMed: 20529254]
53. Yeo PL, Rabenstein DL. Characterization of the thiol/disulfide chemistry of neurohypophyseal peptide hormones by high-performance liquid chromatography. *Anal Chem.* 1993; 65:3061–3066. [PubMed: 8256869]
54. Patai, S.; Rappoport, Z. *The Chemistry of sulphur-containing functional groups.* Wiley & Sons; Chichester: 1993.
55. Nagy P. Kinetics and Mechanisms of Thiol-Disulfide Exchange Covering Direct Substitution and Thiol Oxidation-Mediated Pathways. *Antioxidants & Redox Signaling.* 2013; 18:1623–1641. [PubMed: 23075118]
56. Whitesides GM, Houk J, Patterson MAK. Activation Parameters for Thiolate Disulfide Interchange Reactions in Aqueous-Solution. *Journal of Organic Chemistry.* 1983; 48:112–115.
57. Zavialov AV, Gaestel M, Korpela T, Zav'yalov VP. Thiol/disulfide exchange between small heat shock protein 25 and glutathione. *Biochim Biophys Acta.* 1998; 1388:123–132. [PubMed: 9774716]
58. Holmgren A. Thioredoxin catalyzes the reduction of insulin disulfides by dithiothreitol and dihydrolipoamide. *J Biol Chem.* 1979; 254:9627–9632. [PubMed: 385588]
59. Fernandes PA, Ramos MJ. Theoretical insights into the mechanism for thiol/disulfide exchange. *Chemistry.* 2004; 10:257–266. [PubMed: 14695571]
60. Pappas JA. Theoretical Studies of Reactions of Sulfur-Sulfur Bond .1. General Heterolytic Mechanisms. *Journal of the American Chemical Society.* 1977; 99:2926–2930.
61. Keire DA, Strauss E, Guo W, Noszal B, Rabenstein DL. Kinetics and Equilibria of Thiol Disulfide Interchange Reactions of Selected Biological Thiols and Related Molecules with Oxidized Glutathione. *Journal of Organic Chemistry.* 1992; 57:123–127.
62. Youngman KM, Spencer DB, Brems DN, DeFelippis MR. Kinetic analysis of the folding of human growth hormone. Influence of disulfide bonds. *J Biol Chem.* 1995; 270:19816–19822. [PubMed: 7649991]

Thiol ionization **Reversible thiol-disulfide exchange** **Thiol oxidation** **Scheme 1.**

Reaction schemes for thiol-disulfide exchange in hGH-derived peptides

Thiol ionization **Reversible thiol-disulfide exchange** **Scheme 2.**

Figure 1.

The concentration of reactants and products for a kinetic study with T20 and T20-T21 at pH 7.0, 10 mM PB and 0.08 M ionic strength (without EDTA or N₂ sparging). Initial concentrations of peptides were: [T20] = 350 μM; [T20-T21] = 250 μM. The symbols represent actual data points obtained from samples at different times (n=3): T20 (□), T20-T21 (◆), T20-T20 (□), T21 (•), and T21-T21 (○). Solid lines are model predictions for the reactions in Scheme 1.

Figure 2.

Thiol-disulfide exchange initial rate studies in which the concentration of one reactant (either T20 or T20-T21) was fixed while the concentration of the second reactant (either T20 or T20-T21) was varied (see text). Reaction kinetics was monitored at pH 7.0, 10mM PB, 0.08M ionic strength with EDTA and N₂ sparging at 22 °C. Log-log plots of rate of loss of T20 versus T20 concentration (□) and rate of loss of T20-T21 versus T20-T21 concentration (◊).

Figure 3.

The concentration of reactants and products for a kinetic study with T20 and T20-T21 at pH 7.0, 10mM phosphate buffer with 0.5 mM EDTA and N₂ sparging. Initial concentrations of peptides were: [T20] = 350 μM; [T20-T21] = 250 μM. The symbols represent actual data points obtained from samples at different times (n=3): T20 (□), T20-T21 (◆), T20-T20 (□), T21 (●), and T21-T21 (○). Solid lines are model predictions for the reactions in Scheme 1.

Figure 4.

Comparison of rate constants for reactions with (black bars) and without (light grey bars) oxidation suppressants (0.5 mM EDTA and N_2 sparging) at pH 7.0, 10 mM PB and 0.08 M ionic strength at 22 °C. Initial concentrations of peptides were: $[\text{T20}] = 350 \mu\text{M}$ and $[\text{T20-T21}] = 250 \mu\text{M}$ ($n=3$).

Figure 5. pH-rate profile for second order rate constants k_1 (◆), k_2 (○), k_3 (▲) and k_4 (□) (see Scheme 1 and text for details) obtained for kinetic studies with T20 and T20-T21 at 22 °C. All rate constants are values extrapolated to zero buffer concentration.

Figure 6.

Pseudo-first order rate constant for the loss of T20-T21 (◆) at different pH (10 mM buffer, 0.08 M ionic strength with oxidation suppressants) and at 22 °C. Rate constants were determined by non-linear regression. Uncertainties are within the size of the symbol (n=3).

Figure 7.

Arrhenius plots for microscopic rate constants k_1 ($\blacklozenge$), k_2 ($\blacksquare$), k_3 ($\blacktriangle$) and k_4 ($\boxtimes$) for the reaction of T20 and T20-T21 (Scheme 1) at pH 7.0, 10 mM phosphate buffer and 0.08 M ionic strength with 0.5 M EDTA and N_2 sparging ($n=3$).

Figure 8.

Observed pseudo-first order rate constants for the loss of cT20-T21 and T20-T21 at different concentration ratios and temperatures. Ratios in figure legend represents thiol:disulfide ratio (T20:T20-T21/cT20-T21). Initial concentrations of peptides were: [T20] = 450 μ M; [T20-T21] = 45 μ M for 10:1 studies and [T20] = 350 μ M; [T20-T21] = 250 μ M for the 1.4:1 study. Uncertainties are within the size of the symbol (n=3).

Table 1

Abbreviations and amino acid sequences of hGH-derived peptides used in studies of disulfide scrambling

Abbreviation	Description	Amino acid sequence	Theoretical mass	Observed mass
T20	Monomeric peptide, free SH group	NH ₂ -IVQCR-OH	617.3319	617.3290
T21	Monomeric peptide, free SH group	NH ₂ -SVEGSCGF-OH	784.3062	784.3067
T20-T20	Homodimer, disulfide linked	NH ₂ -IVQCR-OH ⋮ NH ₂ -IVQCR-OH	1232.6482	1232.6247
T21-T21	Homodimer, disulfide linked	NH ₂ -SVEGSCGF-OH ⋮ NH ₂ -SVEGSCGF-OH	1566.5978	1566.5984
T20-T21	Heterodimer, disulfide linked	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

Table 2

Activation parameters for thiol-disulfide exchange reactions and oxidation reaction (k_5). Values for $H^\ddagger$, $S^\ddagger$ and $G^\ddagger$ were obtained from the Eyring plot shown in Supporting Information Figure S5.

rate constant	Ea(kJ/mol)	A	$H^\ddagger$ (kJ/mol)	$S^\ddagger$ (J/mol.K)	$G^\ddagger$ 25 °C (kJ/mol)
k_1	46±4	$7.5 \times 10^8 \pm 4.2^a$	43±6	-83±20	68±9
k_2	41±2	$2.0 \times 10^8 \pm 2.6^a$	38±4	-96±15	66±6
k_3	48±4	$8.9 \times 10^8 \pm 5.9^a$	50±6	-66±21	70±9
k_4	48±4	$2.3 \times 10^9 \pm 4.4^a$	46±5	-75±16	67±7
k_5	53±7	$1.5 \times 10^{12} \pm 16.0^b$	51±6	134±19	11±8

^a units in $M^{-1} s^{-1}$

^b units in $M^{-3} s^{-1}$

Table 3

The change in cT20-T21 and T20-T21 concentrations obtained after 6 hours for thiol-disulfide exchange reactions with T20 at different pH and temperature at thiol: disulfide of 10:1.

Peptide	pH	Temp (°C)	% decrease after 6 hours
cT20-T21	7.0	22	17.3±3.3
	7.0	40	18.6±2.4
	9.0	22	26.0±3.6
	9.0	40	23.6±3.2
T20-T21	7.0	22	79.9±9.8
	9.0	22	86.4±2.9