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Thiol-disulfide exchange in human growth hormone

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Abstract

Purpose—Thiol-disulfide exchange was monitored in recombinant human growth hormone (hGH) and in model tryptic peptides derived from hGH to investigate the effects of higher-order structure on the reaction.

Methods—Different free thiol-containing peptides, varying in length and amino acid sequence, were used to initiate the reaction at pH 7.0 and 37 °C in hGH. Protein samples were digested with trypsin and analyzed for native disulfides, scrambled disulfides and free thiols using LC/MS. The loss of native disulfide and disulfide exchange was compared with model peptides derived from hGH.

Results—Loss of native disulfide in cyclic (cT20-T21) and linear peptides (T20-T21_{pep}) derived from the C-terminal hGH disulfide during the first 60 min of reaction was greater than loss of the C-terminal disulfide in hGH itself. Of the thiols tested, glutathione (GSH) was the most reactive, forming the highest percentage of mixed disulfides in intact hGH and in the model peptides. At longer reaction times (>240 min), native disulfides in both hGH and cT20-T21 were regenerated. The fastest rates of regeneration were observed for Cys and the di- or tripeptide containing an Arg residue adjacent to Cys, suggesting that they may be useful in refolding.

Conclusions—Thiol-disulfide exchange reactions in hGH and related model peptides were influenced by higher order structure, by the size of the thiol reactant and by an Arg residue adjacent to Cys in the thiol reactant. Reduction of disulfide bonds in hGH did not affect higher order structure as measured by CD and HDX-MS.

Keywords

human growth hormone; protein; peptide; kinetics; thiol-disulfide exchange

INTRODUCTION

The formation of correct disulfide bonds is central to the development of safe and efficacious protein drug products. While the perturbation of native disulfide bonds helps to regulate the activity of enzymes such as thioredoxin reductase *in vivo*, disulfide bond cleavage is undesirable in protein drugs, and mismatched disulfides can result in misfolding,

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SUPPORTING INFORMATION

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

aggregation and loss of activity (1). To ensure drug quality and product homogeneity, the presence of scrambled disulfides and the extent of scrambling need to be determined. A detailed mechanistic understanding of thiol-disulfide exchange based on such measurements would reduce attrition rates during drug development and decrease the time-to-market.

The formation of protein mixed disulfides (P-S-S-R) occurs in all biological environments, typically by one of two mechanisms: (i) thiol-disulfide exchange; $P-S-S-P + R-SH \leftrightarrow P-S-S-R + P-SH$ or (ii) sulfenic acid formation; $P-SOH + R-SH \rightarrow P-S-S-R + H_2O$ (2), where R-SH is any low-molecular weight thiol (reduced), P-S-S-P is the native disulfide bond in a protein, P-SH is the protein with a free thiol, P-SOH is protein sulfenic acid and P-S-S-R is the mixed disulfide. Thiol-disulfide exchange reactions are favored at neutral to alkaline pH and the reaction proceeds via an S_N2 nucleophilic displacement. The oxidized forms of low-molecular weight thiols (R-S-S-R) produce protein disulfide bonds via oxidation, while the reduced forms (R-SH) are used to rearrange scrambled disulfide bonds *in vivo*. The leaving group typically has a lower pKa than the central sulfur atom that is attacked by a thiolate anion (2). Intrinsic factors other than thiol pKa also affect thiol-disulfide exchange, including the ionic strength of the medium (3), geometric strain imposed on the disulfide bond by higher-order structure (4) and the relative stability of the native and non-native disulfide bonds (5).

Proteins can form mixed/scrambled disulfides with other thiols, which may be present as impurities or in the protein sequence as a free cysteine (Cys) (6, 7). A sulfur group with better exposure to the attacking nucleophile is more prone to thiol-disulfide exchange than more buried residues (8). These mixed disulfides can then initiate other thiol-disulfide exchange reactions, including the formation of disulfide-linked aggregates. Disulfide bond degradation in therapeutic proteins can also occur via β -elimination (9) and thiol-catalyzed exchange (10) during processing and storage, or after administration on exposure to low-molecular weight thiols in serum (11). In some proteins such as monoclonal antibodies, incomplete disulfide bond formation also produces free +Cys (12) that can then participate in various degradation reactions.

The rearrangement of native disulfide bonds to form mixed disulfides via thiol-disulfide exchange has been reported for a number of therapeutic proteins (10, 13, 14). Disulfide-linked isomers of interleukin-2 (IL-2) were observed in the presence of a chaotrope under alkaline conditions. IL-2 has one free Cys and one disulfide bond in its native form. Alkaline and denaturing conditions deprotonate the free thiol, thus mediating thiol-disulfide exchange reactions that generate less pharmacologically active disulfide-linked isomers (15). Thiol-disulfide exchange reactions have also been observed in monoclonal antibodies. The C_H1 domain of IgG2 contains a free Cys that is involved in the formation of disulfide-linked oligomers (intermolecular disulfides) on agitation (7). While thiol-disulfide exchange is known to occur in proteins and peptides, the effects of protein sequence and higher-order structure on reaction kinetics are not well understood and are the focus of this work.

The studies reported here address the effects of higher order structure and peptide sequence on thiol-disulfide exchange in recombinant human growth hormone (hGH) and peptides derived from it (Fig. 1, Table I). hGH is a commercial therapeutic protein used to treat a

number of growth related disorders, including include Turner's syndrome, Prader-Will syndrome and chronic kidney insufficiency. In 2012, hGH was one of the top 200 pharmaceutical products by sales in the U.S. (16). Although hGH has no free Cys, a free thiol group maybe generated via β -elimination (9, 17) and can facilitate disulfide exchange and lead to loss of therapeutic efficacy. hGH was selected as the model protein for these studies due to its relatively small size and the availability of cDNA for hGH expression in-house. Our familiarity with thiol-disulfide exchange in model peptides derived from hGH was an additional advantage (18, 19). Native hGH contains two disulfide bonds (Cys53-Cys165, Cys182-Cys189); our focus is the C-terminal disulfide (Cys 182-Cys 189).

To facilitate the analysis of thiol-disulfide exchange in hGH, a liquid chromatography / mass spectrometry (LC/MS) method with tryptic digestion was developed that minimizes scrambling during digestion and analysis. LC/MS methods have emerged as important analytical tools for characterizing protein structure and mapping disulfide bonds (20, 21). The analysis of disulfide bonds using a bottom-up approach can be challenging, however, particularly when using tryptic digestion, since trypsin is most active at alkaline pH and high temperature, conditions that also favor disulfide scrambling. While digestion with pepsin has been reported to minimize disulfide scrambling (22), trypsin was used here to allow reproducible quantitation of scrambled species using synthetic tryptic-peptide standards. Accordingly, we report an LC/MS approach using tryptic digestion without reduction, followed by chromatographic separation, for the qualitative and quantitative analysis of native and non-native disulfide-linked peptides in hGH.

MATERIALS AND METHODS

Materials

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 , ethylenediaminetetraacetic acid (EDTA), Trizma (Tris.HCl), urea, sucrose, glutathione (oxidized-GSSG), glutathione (reduced-GSH), L-cysteine (Cys, C) and glycerol 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. A flash digest kit (trypsin) for protein digestion was obtained from Perfinity Biosciences (West Lafayette, IN). Human growth hormone (1 mg/mL) was purchased as a lyophilized powder (0.34 mg phosphate buffer and 8 mg mannitol) from ProSpec-Tany TechnoGene Ltd., Ness Ziona, Israel.

Synthetic peptides were used as models of the C-terminal disulfide bond in hGH (Figure 1), as free thiol-containing reagents in thiol-disulfide reactions with hGH, and as standards for analysis of hGH scrambling products (Table I). Peptides cT20-T21 and T20-T21_{pep} ("Disulfide-linked peptides", Table I) are the cyclic and linear forms of the C-terminal disulfide bond in hGH (i.e., Cys182-Cys189), respectively; we have reported the kinetics and mechanisms of thiol-disulfide exchange reactions in these peptides previously (18, 19). Comparing the rates of reaction of these synthetic peptides (cT20-T21, T20-T21_{pep}) with

rates in intact hGH allows the effects of higher order structure on reactivity to be inferred. Thiol-containing peptides (“Free thiol-containing peptides”, Table I) are reactants and/or products in thiol-disulfide exchange reactions with hGH and model peptides. A sequence modified form of T20 with an additional N-terminal Gly residue (T20a; Table I) was included to enable reactants and products to be distinguished in reactions of T20a with hGH. Additional free thiol-containing peptides (GSH, RCR, QCR, CR; Table I) were used to probe the effects of primary sequence on reactivity. The remaining peptides (“Peptides from Trypsin digestion”, Table I) are the products of thiol-disulfide exchange reactions of hGH with GSH (T20-S-S-G, T21-S-S-G, T6-S-S-G, T16-S-S-G; Table I) and with T20a (T20a-T20, T20a-T21, T20a-T6, T20a-T16; Table I), and were used as synthetic standards to enable the identification and quantitation of these reaction products, with a focus on quantitation of T20 and T21 products. All peptides were purchased from GenScript, (Piscataway, NJ) with >90% purity as lyophilized powders. Hereinafter, three-letter amino acid abbreviations are used in the text to ensure readability, while one-letter abbreviations are used in tables for brevity. Where there is any ambiguity in the text, both forms are included.

Methods

Expression and purification of hGH—The plasmid containing the hGH expression gene was obtained from Dr. Jennifer Laurence (University of Kansas, Lawrence, KS). This plasmid codes for hGH with an additional 28 amino acids at the N-terminus and has a molecular weight of 25 kDa. The plasmid was transformed into BL21 (DE3) *E.coli* cells for protein expression, using the protocol provided by New England BioLabs. Cells carrying the plasmid for hGH were grown in terrific broth medium containing 100 µg/mL ampicillin in an incubator shaker at 37 °C. The cells were induced at OD₆₀₀ = 0.8 with 1 mM IPTG. Post-induction was carried out in the incubator shaker at 20 °C for 16 hours. The expression of hGH in the induced cells was confirmed using 12% SDS-PAGE and the protein was found to be overexpressed as inclusion bodies (Fig. S1a).

hGH was purified using a method reported previously (23). Briefly, induced *E.coli* cells were harvested by centrifugation at 6500 rpm for 15 min. The cell pellet was resuspended in 50 mM Tris-HCl, 100 mM NaCl, 5 mM EDTA at pH 8.0 and sonicated to lyse the cells. rhGH inclusion bodies were separated from the soluble cell lysate by centrifugation at 13,000 rpm for 30 min. The pellet with cell debris was then resuspended in buffer, 50 mM Tris-HCl, 5 mM EDTA, 1% deoxycholate (DOC) at pH 8.0, sonicated and centrifuged to further clarify the inclusion bodies. The pellet was washed with buffer, 50 mM Tris-HCl at pH 8.0 and centrifuged to remove any DOC from the previous step. Purified inclusion bodies were solubilized in buffer containing 100 mM Tris-HCl and 2 M urea at pH 12.5. The solution was further diluted 10 times in a buffer containing, 50 mM Tris-HCl, 2 M urea, 0.5 mM EDTA, 3 mM GSH, 0.6 mM GSSG, 10% glycerol and 2% sucrose at pH 8.0. Refolding was carried out by step-wise dialysis of the protein solution against buffer (50 mM Tris-HCl, 0.5 mM EDTA, 10% glycerol, 2% sucrose; pH 8.0) by decreasing the concentration of urea from 2–0 M at each step (1.5, 0.5 and 0 M). The refolded rhGH was filtered and purified further using a HiPrep 26/60 Sephacryl S-100 high resolution column (Amersham Biosciences, Piscataway, NJ) equilibrated with 50 mM Tris-HCl, 2% sucrose at pH 8.0 and

the fraction containing monomeric hGH was confirmed using 12% SDS-PAGE and its purity assessed (Fig. S1b). The concentration of purified hGH was determined using a UV-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA). MS analysis of the purified protein confirmed the presence of two native disulfide bonds as found in the commercially available hGH (Fig. S2).

Thiol-disulfide exchange in hGH—The kinetics and products of thiol-disulfide exchange reactions occurring with hGH and various thiol-containing peptides (T20a, GSH, RCR or CR; Table I) were investigated. To initiate the reaction, 280 μ L of hGH purified in-house (0.56 mg/mL or 22.4 μ M) in 50 mM Tris.HCl buffer and 2% sucrose (pH 8.0) were mixed with 70 μ L of the thiol (8950 μ M in 0.1% FA in DDI). The pH of the final reaction mixture was 7.0 and the final molar ratio of peptide to protein was 100:1 (1790 μ M: 17.9 μ M). 90 μ L aliquots of the reaction mixture were then transferred to microfuge tubes (n=3 for each time point) and placed in an incubator at 37 °C. Samples were withdrawn in triplicate at various times, desalted, digested and analyzed using LC/MS as described below. Control samples containing hGH alone were diluted, desalted and digested as for the reaction samples at other time points. To determine contributions from the additional 28 amino acids in the modified hGH expressed in-house, the reaction of Prospec hGH with GSH was also monitored. Prospec hGH was first buffer exchanged (overnight at 4 °C) into 50 mM Tris.HCl and 2% sucrose before reaction with GSH. Sample digestion and analysis were carried out as described below.

Removal of unreacted peptides using a desalting column—At each time point, samples were desalted to remove unreacted peptide and to minimize scrambling during subsequent digestion and analysis. Zeba™ spin desalting columns with 7K MWCO (Pierce Biotechnology, Rockland, IL) were used for this purpose. The spin columns were first placed in an empty collection tube (1.5–2 mL) and centrifuged at 4700 rpm using an Eppendorf centrifuge (Eppendorf, Hauppauge, NY) with the F45–11–12 rotor for 1 min to remove the storage buffer. Reacted samples (80–85 μ L) were then added carefully to the center of the resin bed. After the sample had adsorbed onto the resin, the spin columns were placed inside new collection tubes and centrifuged at 4700 rpm for 2 min. The desalted sample was collected and digested as described below prior to LC/MS analysis.

Trypsin digestion using the flash digest protocol—A Perfinity flash digest kit was used to digest hGH samples prior to LC/MS analysis, hGH-containing samples were added to the Perfinity flash digest kit tubes using the digestion buffer provided and a sample: buffer ratio of 1:2.4. Digestion was performed at 60 °C and 1400 rpm using a ThermoMixer C (Eppendorf, Hauppauge, NY) for 15–17 min. Digested samples were then transferred to microcentrifuge tubes and centrifuged at 14,000 rpm for 2 min. After centrifugation, the supernatant was removed, quenched with 10 μ L of 20% FA in DDI and analyzed using LC/MS (~20 μ L). In preliminary studies, Perfinity digestion was compared with: (i) overnight digestion with trypsin in solution, (ii) digestion with trypsin immobilized on magnetic beads and (iii) digestion with trypsin immobilized on agarose beads. For all digestion protocols except the Perfinity flash digest, scrambled disulfides were detected and digestion was incomplete after 60 min. In contrast, digestion with the flash digest kit was

complete in 17 min and no scrambled disulfides were detected. The Perfinity flash digest kit was therefore used in all the studies reported here.

LC/MS analysis—Mass analyses of hGH digests were carried out using an LC/MS system (1200 series LC, 6520 qTOF, Agilent Technologies, Santa Clara, CA). Peptides in the digested samples were separated prior to MS analysis using a Zorbax 300SB-C18 column from Agilent Technologies. Solvent A was 0.1% FA in water and solvent B was 0.1% FA in ACN. A gradient run followed this time course: 5% B held at 5% for 5 min; increase to 20% B in 8 min; increase to 25% B in 10 min; increase to 60% B in 5 min; increase to 100% B in 0.6 min; decrease to 0% B in 0.6 min; return to 5% B in 2.4 min. The flow rate was maintained at 50 μ L/min and the column temperature was not controlled. A representative total ion chromatogram (TIC) for tryptic digest of hGH is shown in Fig. S2. For LC/MS analysis, a 20 μ L sample was injected. To determine native and scrambled disulfide species, a library of all peptides containing a disulfide bond and/or free Cys was generated. Briefly, the amino acid sequence of hGH was entered into the MassHunter software (Agilent) and an *in silico* digest was performed using trypsin as the enzyme and with the number of missed cleavages set to four to generate a list of all possible tryptic fragments. From this list, all peptides with disulfide bonds and/or free Cys were selected and combined to create a mass filter list. Mass spectra of all digested hGH samples were then scanned against the mass filter list to determine the presence of scrambled disulfides and the extent of disulfide scrambling. The loss of native disulfide (T20-T21) was monitored at different reaction times using peak areas from extracted ion chromatograms (XIC); the concentration was calculated using a calibration curve constructed with a peptide standard from GenScript (Piscataway, NJ). Similar calibration curves were constructed for T20 and T21 using peptide standards (GenScript).

Near-UV CD measurement—Near-UV CD spectroscopy was used to assess the effects of disulfide reduction on the tertiary structure of hGH. Samples with and without 5 mM β -mercaptoethanol were diluted to 2 μ M final hGH concentration. Molar ellipticity was measured on a JASCO J-815 spectrometer (JASCO Analytical Instruments, Easton, MD) in a 10 mm path length quartz cuvette. Spectra were acquired for wavelengths of 250 nm to 350 nm at a scanning speed of 50 nm/min.

Amide hydrogen-deuterium exchange mass spectrometry (HDX-MS)—HDX-MS was conducted to determine the solvent accessibility of hGH under native and reduced conditions. A 2 mg/mL solution of hGH was prepared in buffer containing 10 mM sodium phosphate, 1.6 % mannitol, pH 7.0 (buffer A). To obtain reduced hGH, a 2 mg/mL solution was prepared in buffer containing 10 mM sodium phosphate, 1.6 % mannitol, 10 mM DTT, pH 7.0 (buffer B) and incubated on ice for 1 h. HDX was initiated by mixing 3 μ L of the sample with 27 μ L of deuterated buffer A and buffer B for native and reduced hGH, respectively. Exchange was carried out at 23 $^{\circ}$ C for 10 s to 10 min. Reactions were quenched by adding 30 μ L of ice cold buffer containing 0.2 M sodium phosphate, 0.5 M Tris(2-carboxyethyl)phosphine hydrochloride, 6 M guanidine hydrochloride to a final pH of 2.5. Deuterium uptake was measured using the LC/MS system described above and equipped

with a custom-built column refrigeration unit (0 °C) to control column temperature and minimize back exchange.

Approximately 50 pmol of protein was injected onto to an immobilized pepsin column. The digests were desalted and trapped in a peptide microtrap (Michrom Bioresources, Auburn, CA) with 10% ACN, 90% water, and 0.1% FA for 4 min. Peptides were eluted onto a reverse phase analytical column (Zorbax 300SB-C18; Agilent Technologies, Santa Clara, CA) for 4.3 min using a gradient to 60% ACN, 40% water, and 0.1% FA. Mass spectra were acquired in the m/z range 200–1700. Peptic digests of undeuterated hGH were analyzed and their masses identified using the MassHunter software (Agilent). Peptides identified from undeuterated hGH were mapped onto data from deuterated samples using HDExaminer software (Sierra Analytics, Modesto, CA) to determine the extent of deuterium uptake. The analysis times for all HDX samples were similar, so that the observed deuterium uptake values were not subjected to back-exchange corrections. Data from 5 min HDX were mapped onto the crystal structure of hGH (PDB ID: 1 HGU) using PyMOL software (PyMOL Molecular Graphics System, Version 1.3, Schrodinger, LLC).

Thiol-disulfide exchange in model peptides—To probe the effects of higher order structure, the reaction was also monitored in cyclic and linear peptides derived from the Cys182-Cys189 disulfide bond. Disulfide-containing peptides cT20-T21 and T20-T21_{pep} (Table I) were reacted with various free thiol-containing peptides (T20a, T20, GSH, RCR, QCR, CR or C; Table I) in aqueous solution (pH 7, 37 °C) and subjected to HPLC analysis to quantify reactants and products. Stock solutions were prepared in 0.1% formic acid in DDI (pH ~ 2.5) to minimize oxidation of free thiols. To initiate the reaction, 1500 μ L of thiol (12,500 μ M), 1500 μ L of T20-T21_{pep} or CT20-T21 (125 μ M) and 750 μ L of 50 mM phosphate buffer (0.08 M ionic strength, 0.5 mM EDTA) were added to a 15 mL BD falcon tube and mixed by pipetting. A 100 μ L aliquot was removed and quenched with 10 μ L 20% formic acid in DDI to verify initial concentrations ($t=0$ min) and the final pH of the reaction mixture adjusted to 7.0. Samples were withdrawn in triplicate at different times, quenched with 20% FA, analyzed and quantitated using RP-HPLC at 215 nm as described previously (18). Calibration curves were linear in the following concentration ranges: 5–500 μ M (T20-T21_{pep}), 20–200 μ M (T20), 20–200 μ M (T21), 5–500 μ M (cT20-T21) and 5–500 μ M (rT20-T21).

As noted above, peptide T20a was used as a surrogate for T20 to allow reactant and leaving group peptides to be distinguished (see Table I). To confirm that the reactivities of T20a and T20 are similar and to investigate buffer effects, the reaction of T20a with T20-T21 (thiol: disulfide = 10: 1) was monitored in 10 mM phosphate buffer (PB) and in 50 mM Tris HCl with 2% sucrose at 22 °C. The final pH of the reaction mixture was 7.0 and samples were analyzed as described previously (18, 19). Pseudo-first order rate constants (k_{obs}) for the loss of T20-T21 were similar: (i) for the reaction with T20 and with T20a, confirming the use of T20a as a surrogate for T20 and (ii) in PB and in Tris HCl with 2% sucrose (Fig. S3), confirming that reactivity is similar in the two solutions.

RESULTS

Thiol-disulfide exchange in hGH: product identification

For the reaction of hGH with T20a (thiol: disulfide = 100:1, molar ratio), four scrambled disulfides; T20a-T20, T20a-T21, T20a-T6 and T20a-T16 (Table I) and four free thiols; T6, T16, T20 and T21 (Table S1) were identified. No peptides with scrambled inter-molecular disulfide bonds (e.g., T20-T20) or non-native intra-molecular disulfide bonds (e.g., T20-T16) were detected at any time. GSH is commonly used in protein refolding and forms mixed disulfides with proteins (2, 24). In the reaction of hGH with GSH, the expected mixed disulfides (T20-S-S-G, T21-S-S-G, T6-S-S-G, T16-S-S-G; Table I) and free thiols (T20, T21, T6, T16) were detected. Interestingly, in the reaction of hGH with CR, one of the mixed disulfides (T20-CR) and several of the free thiols (T6, T16 and T21) were not detected in any of the samples. All the other mixed disulfides, T21-CR, T6-CR and T16-CR and T20 were detected, however (Table S2). In the reaction of hGH with RCR, mixed disulfides (T6-RCR, T16-RCR and T21-RCR) and free thiols (T20 and T6) were detected (Table S2). With Cys (C) as the thiol reagent, only T21-C and T20 were detected (Table S2); mixed disulfides (T20-C, T6-C and T16-C) and free thiols (T6, T16 and T21) were not detected. That scrambling was detected only at the C-terminal disulfide bond in the reaction with Cys (C) may be due to rapid regeneration of the native disulfides, so that the mixed disulfides are not observed at the reaction times monitored here. Products consistent with intermolecular disulfide scrambling (e.g., T20-T20) and products of thiol oxidation (e.g., sulfenic, sulfuric and/or sulfonic acid) were not detected with any of the thiol reagents at any time.

Control samples containing hGH alone (37 °C) and analyzed at 60 mins and 1 day did not show scrambled species (data not shown), indicating that disulfide exchange does not occur under the conditions investigated here in the absence of a free thiol group. The observed change in scrambled species over time for hGH (Figs. 1a, b) and the similarity of this profile with that of the cyclic peptide (cT20-T21, Fig. 4, Fig. S8) suggest that scrambling during digestion (which was used for hGH but not for cT20-T21) and analysis is minimal with the desalting and digestion protocols used. When the desalting column was not used, all possible scrambled disulfides in hGH (Table S1) were identified at all time points. Similarly, scrambled species were also detected at all time points when digestion was performed using other protocols (see Materials and Methods).

Thiol-disulfide exchange in hGH: product quantitation

Thiol-disulfide exchange in hGH was quantified for the C-terminal disulfide bond (Cys182-Cys189; T20-T21). Quantitative analysis of disulfide scrambling for the second hGH disulfide bond (Cys53-Cys165; T6-T16) was not performed due to the lack of peptide standards (i.e., for T6-T16, T6 and T16) and the low aqueous solubility of T6. At the C-terminal disulfide bond (T20-T21), the concentrations of T20-T21, T20 and T21 were determined using XIC peak areas and calibration curves prepared using synthetic peptide standards. The total concentration of mixed disulfides was calculated from mass balance: $[\text{Mixed disulfides}] = [\text{T20-T21}]_{\text{total}} - ([\text{T20-T21}]_t + ([\text{T20}] + [\text{T21}])/2)$, since synthetic standards were not available. In this calculation, it was assumed that disulfide bond cleavage

of 1 mol of T20-T21 releases 1 mol of T20 and 1 mol of T21 that are present either in free thiol or in mixed disulfide form, an assumption supported by the lack of intermolecular scrambling and oxidation products, as noted above. The loss of native disulfide is reported as a percentage of the initial value, as determined from [T20-T21] in the control sample (i.e., at $t = 0$) and [T20-T21] at time, t .

The kinetic profiles for the reaction of hGH with T20a and with GSH are shown in Figs. 2a and 2b; kinetic profiles for the other thiols are available in SI (Fig. S4). In the reaction with T20a (Fig. 2a), mixed disulfides and free thiols were formed before the first sampling time, with a corresponding loss of approximately 50% of the native disulfide (T20-T21). The native disulfide then slowly reformed, so that 60–75% of the initial T20-T21 concentration was detected at the end of the study. Free thiol groups were detected at low levels ($< 0.1 \mu\text{M}$) early in the time course ($t < 1500 \text{ min}$), but not at later times. In the reaction of hGH with GSH (Fig. 2b), mixed disulfides were formed initially at even higher levels than in the reaction with T20a; concentrations at the first time point account for nearly 70% of the initial T20-T21 concentration. Free thiol concentrations were also greater at early time points than in the reaction with T20a, and the native disulfide (i.e., T20-T21) concentration at the first time point was less than 30% of the initial value. The native hGH disulfide again slowly reformed, so that at the end of the time course ~80% of the initial T20-T21 was present. A similar product profile was obtained for Prospec hGH (22kDa) for the reaction with GSH (Fig. S5), suggesting that the leader sequence at the N-terminus in the hGH expressed in-house (25 kDa) does not influence thiol-disulfide exchange appreciably.

To summarize the initial loss and subsequent reformation of the native hGH disulfide (T20-T21) for the various thiols tested, we report the percent decrease in the native disulfide after 60 min and 1 day (1440 min) of reaction (Table II). As in the reaction with T20a and GSH (Fig. 2), the loss in native hGH disulfide at 60 min was greater than or equal to the loss at 1 day for all thiols tested (Table II). In general, the loss of native hGH disulfide at both times decreased somewhat with decreasing size of the thiol group, so that losses were greatest for T20a and least for Cys (C). GSH is an exception to this trend, and showed greater losses at 60 min than T20a despite its smaller size. These losses were less in RCR than in GSH, though both are tripeptides, suggests that the presence of an Arg residue adjacent to Cys favors regeneration of the native disulfide.

Effect of hGH structure on regeneration of native disulfide

To measure the extent of loss of hGH native structure upon disulfide bond cleavage, and to relate the regeneration of native disulfides following thiol-disulfide exchange to structural changes, near-UV CD spectroscopy and amide HDX-MS were conducted for hGH in oxidized and reduced states. Near-UV CD showed no appreciable differences in tertiary structure between the native and reduced forms (Fig. S6). In HDX-MS, a total of 31 peptic peptides covering 100% of hGH sequence were identified and analyzed. Comparison of the level of deuterium uptake between the native and reduced states showed no appreciable differences (Fig. S7), suggesting that hGH retains its native structure after disulfide bond cleavage. The C-terminal peptide fragment, 182–191 containing the two cysteine residues (Cys182 and Cys189) involved in disulfide formation is ~ 50% deuterated in both the native

and reduced states. Similarly, peptide fragment 47–53 containing Cys53 and fragment 160–172 containing Cys165 showed 53% and 38% deuterium uptake, respectively. Subtractive analysis of peptide fragments 160–172 and 166–175 shows that residues 160–165 are more than 60% deuterated. Overall, the near-UV CD and HDX-MS results suggest that the disulfide containing regions in hGH are highly solvent accessible and that the structure of fully reduced hGH is similar to the native state. This suggests that the regeneration of native disulfide bonds following disulfide exchange may be favored by the orientations of the Cys partners in close proximity to one another in the reduced form.

Thiol-disulfide exchange in model peptides

To further investigate the effects of higher order structure on thiol-disulfide exchange, free thiols were reacted with disulfide-containing peptides corresponding to the C-terminal disulfide bond of hGH (Cys182-Cys189). Comparing the loss of the native disulfide in the relatively unstructured model peptides with that in hGH gives a quantitative measure of the effects of higher order structure on the reaction. Thiol-disulfide exchange was monitored in the cyclic peptide cT20-T21 and in the linear peptide T20-T21_{pep} at pH 7.0 and 37 °C. For cT20-T21, the concentrations of mixed disulfides (single and double mixed disulfides, Scheme 1) were determined from mass balance; [Mixed disulfides] = [cT20-T21]_o – ([cT20-T21]_t + [rT20-T21]_t), since synthetic peptide standards were not available. For T20-T21_{pep}, the mass balance equation described above for hGH was used.

In the reaction of the cyclic peptide cT20-T21 with GSH, mixed disulfides and the reduced form (rT20-T21) were detected and quantified (Fig. 3a). Loss of the native disulfide (cT20-T21) was rapid; concentrations decreased to < 15% of the initial value in 60 min (Fig. 3a), with the reduced form as the major product. Over the 2-day reaction time course, the reduced form was slowly reconverted to cT20-T21, so that the final cT20-T21 concentration was ~ 40% of the initial value. Concentrations of mixed disulfides plateaued at ~250 min and were unchanged thereafter. In the reaction of the linear peptide T20-T21_{pep} with GSH, mixed disulfides (T20-S-S-G, T21-S-S-G) and free thiols (T20, T21) were quantified (Fig. 3b). Trace amounts of the homodimers T20-T20 and T21-T21 were also detected but were below the limit of quantitation. At the end of the 2-day reaction time course, the parent disulfide T20-T21_{pep} was not detected, unlike studies with cT20-T21 and hGH in which the native disulfides were regenerated. The reaction of T20-T21_{pep} produces two products, each with a single thiol group, while reactions of cT20-T21 and hGH produce two thiol groups in the one product molecule (Scheme 1). It is expected that reformation of the disulfide in cT20-T21 and hGH is favored by the spatial proximity of the thiol groups imposed by the peptide backbone, but not in T20-T21_{pep} where this constraint is lacking.

Comparing the percent decrease in the native disulfide in cT20-T21 with that in hGH after 60 min and 1 day (1440 min) of reaction provides a quantitative measure of the effects of higher order structure on the reaction (Table II; see Fig. S8 for full kinetic profiles). T20-T21_{pep} was not included in this comparison because, unlike reactions with cT20-T21 and hGH, the native disulfide was not reformed. After both 60 min and 1 day of reaction, the percent loss in native disulfide was greater for cT20-T21 than for hGH for every thiol group tested. At 60 min of reaction, the differences in the percent native disulfide lost (i.e., cT20-

T21 vs. hGH, Table II) ranged from 13 to 45%, and were greatest for CR and C. At 1 day of reaction, the differences in the percent native disulfide lost ranged from 1 to 53%, and were greatest for GSH and least for CR and C. These results suggest that the forward reaction is hindered by higher order structure in hGH and/or the reverse reaction favored, despite the fact that the T20-T21 disulfide bond is solvent-exposed.

DISCUSSION

In the studies reported here, thiol-disulfide exchange reactions between intact hGH or model peptides (cT20-T21 and T20-T21_{pep}) and free thiol-containing peptides (Table I) were investigated in aqueous solution at pH 7.0 and 37 °C. For the reaction of hGH with different thiol-containing peptides, products generally conforming to Scheme 1 were detected, and the distribution of these species varied with reaction time and the type of thiol-containing peptide. Mixed disulfides were detected at both the disulfide bonds (T20-T21 and T6-T16) and fully reduced forms of the native disulfides (Table S1) were also observed. The initial reactivity of the C-terminal native disulfide (T20-T21) in hGH decreased in the order GSH>T20a>RCR>CR≈C and regeneration of native disulfides was favored over the formation of mixed disulfides in the order C≈CR>RCR>GSH>T20a (Table II). Differences in reactivity among the thiol-containing peptides were large. The difference in the percent native disulfide lost at 60 min between the most (GSH) and least (C) reactive thiol groups was more than 40%; the difference in the percent native disulfide lost at 1 day between the most (C) and least (T20a) regenerative thiol groups was more than 30% (Table II). Most of the native disulfide was regenerated in the presence of GSH (75–80%), RCR (85–90%), CR (92–95%) and C (94–99%), suggesting that these thiols may be effective refolding agents. The presence of Arg adjacent to Cys appears to promote reformation of the native disulfide (Table II). To a lesser extent, smaller amino acid chain length also appears to promote refolding (Table II), though the range of chain lengths examined here (1–5 amino acids) was relatively small. It should be noted that the presence of even low levels of mixed disulfides may be detrimental to the therapeutic efficacy and stability of hGH, however, given the functional roles of the two native disulfide bonds. The C-terminal disulfide bond (T20-T21) is required for binding to the growth hormone receptor and to maintain normal stability, while the disulfide bond in T6-T16 is necessary for biological activity (25).

Mixed disulfides and rT20-T21 were identified as products of the reaction of cT20-T21 with all the free thiol-containing peptides (Table I). Oxidation products such as cysteine sulfenic, sulfinic and sulfonic acid and thiosulfates were not detected, and Scheme 1 effectively describes the products observed. Overall, the loss of native disulfide in cT20-T21 after 60 min decreased in the order GSH>T20>CR>T20a>QCR>C>RCR and regeneration of native disulfide was favored after 1 day in the order CR>C>RCR>QCR>T20a>T20>GSH. Comparison of the percent decrease in the native disulfide in hGH and in cT20-T21 gives a measure of the effects of higher order structure. At both 60 min and 1 day of reaction, the percent decrease in native disulfide was greater in cT20-T21 than in hGH for every thiol tested (Table II). At 60 min, the differences in percent lost (i.e. (% native disulfide lost for cT20-T21) – (% native disulfide lost for hGH)) ranged from 14 to 41%, and were generally greater for the smaller thiol groups. At 1 day, the differences in percent lost ranged from 1 to 53%, and were greatest for GSH. The magnitude of these effects is similar to that of the thiol

group type (Table II), suggesting that both are important in the reaction in hGH. Interestingly, higher order structure plays a role even though the disulfide bond studied (Cys182-Cys189; T20-T21) is highly solvent exposed, perhaps because retention of structure in reduced hGH helps promote reformation of disulfide bonds.

Native disulfides in hGH were re-formed in the presence of GSH, RCR, CR, C and to a lesser extent with T20a. Rabenstein et al. made a similar observation with somatostatin and GSH. At concentrations of GSH less than 4 mM, Rabenstein et al observed that the rate of reformation of the native disulfide was faster than the rate of thiol-disulfide exchange to form the fully reduced form (26). The authors also concluded that the conformational properties of the mixed disulfides may place the mixed disulfide in close proximity to the thiol group that then regenerates the native disulfide. Similar results have been reported for the reaction of small heat shock protein 25 (Hsp25) with GSH, where the formation of an intersubunit protein disulfide (native disulfide) is preferred over the mixed disulfides (24). Here, near-UV CD and HDX-MS of both reduced and non-reduced hGH (Figs. S7, S8) showed no differences in higher order structure, thus favoring regeneration of native disulfides. Youngman et al. have shown that folding of hGH is similar in the presence and absence of disulfide bonds (27), further suggesting that disulfide bond reduction does not affect protein structure.

Regeneration of native disulfides was fastest in the presence of RCR, CR and C (Table II). For the reaction of hGH with Cys (C), formation of mixed disulfides was minimal and most of the native disulfide remained intact after 60 mins and 1 day. Previously, Raman et al. have shown that the Cysteine/Cystine redox system is more efficient in the oxidative folding of high concentrations (0.05 mg/mL) of lysozyme than GSH/GSSG (28). Though only Cys was added in the studies here, oxidation of Cys over time can result in the formation of Cystine thus providing a Cysteine/Cystine redox system. This observation with lysozyme and our results suggest that Cys favors the formation of native disulfides over mixed disulfides, and that the rate of formation of mixed disulfides is much less than the rate of regeneration of native disulfides, may explain the detection of only one of the mixed disulfides with Cys (C) in our studies (i.e., T21-C). In addition, the thiol group in Cys itself has a low pKa (8.3) that would make it a better leaving group than the central Cys.

With RCR and CR, the presence of a positively charged group adjacent to the Cys (C) may increase the reactivity of the thiol group by lowering thiol pKa. A thiol group with low pKa makes a better leaving group (2), since the charge stabilizes the thiolate form over the mixed disulfide (29), and thus favors reformation of native disulfides in both cT20–21 and hGH. The presence of Arg (R) adjacent to Cys 182 in growth hormone is highly conserved among different species; the positively charged residue lowers the pKa of the thiol group making it the most reactive of the four Cys residues (30). Having two Arg residues flanking the reactive Cys did not increase reactivity over having just one, however; similar reactivities of CR and RCR were observed for cT20-T21 (Fig. 4). However, with QCR, the regeneration of the native disulfide is slower than with RCR and CR (Fig. 4). In agreement with the results obtained here, Okumura et al. observed that with a glutathione derivative, RCG (oxidized and reduced), prouroguanylin folded into its native conformation more efficiently than with

GSH (31). The increase in folding efficiency was attributed to the presence of a positive charge (from Arg (R)) adjacent to the Cys (C) residue.

Redox potentials for the different thiol-containing peptides (Table I) were not determined because their oxidized forms were not available and measurement of redox potentials (e.g., by cyclic voltammetry) is beyond the scope of this work. To gain a better understanding of the observed peptide reactivities towards the native disulfides in hGH, a unified software package DiANNA was used to estimate the Cys reactivity state in the free thiol-containing peptides. The algorithm predicts the susceptibility of Cys residues to either participate in a disulfide bond or to be in the reduced state with 76–81 % accuracy (32). Scores obtained for the different peptides used to initiate thiol-disulfide exchange are shown in Table S3; a higher score corresponds to an increased susceptibility to be in that state. The lowest scores for “half-Cys” (i.e., the thiol form) were obtained for C and CR, suggesting that in the presence of these thiols, regeneration of the native disulfide will be favored over mixed disulfides, in agreement with the results obtained here (Fig. 4). However, similar half-Cys scores were obtained for RCR, QCR and GSH and no correlations could be made to the results reported here. rT20-T21, the reduced form of cT20-T21, had the highest half-Cys score, suggesting that the disulfide bond in cT20-T21 would be favored over its reduced form or a mixed disulfide with another free thiol-containing peptide. The differences in peptide reactivity between DiANNA scores and the reactivities reported here suggest that, in addition to the influence of primary structure on thiol pKa, higher order structure also plays a role and can determine the type of disulfide bond that can be regenerated.

CONCLUSIONS

In thiol-disulfide exchange reactions in hGH and related model peptides, reactivity was influenced by: (i) protein higher order structure, with a 13–45% greater loss in the native disulfide reacted (60 min) in a cyclic peptide (cT20-T21) than in hGH (Table II), (ii) by the size of the thiol reactant, with reduced loss of native disulfides (60 min) for smaller thiol reactants (Table II), and (iii) by an Arg residue adjacent to Cys in the thiol reactant, which showed reduced loss in native disulfide (60 min, 1 day), probably due to the effect of Arg on the Cys pKa. Reformation of the native disulfide bond in hGH at longer reaction times was favored by Cys (C) and by tripeptides containing an Arg residue adjacent to Cys, suggesting that they may be useful as refolding reagents. To enable these studies, a protocol for tryptic fragmentation of hGH that minimizes disulfide scrambling during digestion was developed. Reduction of disulfide bonds in hGH did not affect higher order structure as measured by CD and HDX-MS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

ACN	acetonitrile
Cys	cysteine
DTT	dithiotreitol
FA	formic acid
GSH	glutathione (reduced form)
hGH	human growth hormone
LC/MS	liquid chromatography / mass spectrometry
MWCO	molecular weight cut-off
PB	phosphate buffer
qTOF	quadrupole time-of-flight
TIC	total ion chromatogram
XIC	extracted ion chromatogram

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Figure 1. Amino acid sequence of human growth hormone (hGH). Tryptic cleavage sites are indicated by arrows; tryptic fragments T1 to T21 are numbered sequentially from the N-terminus. Fragments T20 and T21 are the focus of this work and are shown in red and green, respectively.

Figure 2.

Concentrations of T20-T21 (◆), T20 (■), T21 (●) and mixed disulfides (T20a-T20+T20a-T21 or T20-S-S-G+T21-S-S-G) (▲) for the reaction of hGH with T20a (A) and GSH (B) at pH 7.0 (40 mM Tris.HCl and 1.6% sucrose) and 37 °C (n=3, +/- SD). The initial molar ratio of thiol: hGH was 100:1. Concentration of hGH alone before reaction with T20a is represented by . Lines on plot are to improve readability and do not represent regression analysis.

Figure 3.

Concentrations of species for the reaction of cT20-T21 or T20-T21_{peg} with GSH at pH 7.0 (10 mM phosphate buffer) and 37 °C (n=3, +/- SD), initial molar ratio of GSH: cT20-T21 or T20-T21_{peg} was 100:1. (A) Concentrations of cT20-T21 (◆), rT20-T21 (■) and mixed disulfides (▲). (B) Concentrations of T20-T21_{peg} (◆), T20 (■), T21 (●) and mixed disulfides (▲). T=0 min represents concentration of native disulfide (cT20-T21 or T20-T21_{peg}) before initiation of reaction. Lines on plot are to improve readability and do not represent regression analysis.

Figure 4.

Kinetics of loss of cT20-T21 (n=3, $\pm$ SD) during the reaction with GSH (■), CR (▲), RCR (●), QCR (□) and C () (pH 7.0, 37 °C, 10 mM PB, 0.08 M ionic strength, 0.5 mM EDTA, N₂ sparged). Initial thiol: disulfide was 100:1 (molar ratio). Lines on plot are to improve readability and do not represent regression analysis.

Scheme 1.

Reaction scheme showing reversible thiol-disulfide exchange between the solvent exposed disulfide in hGH or a related model peptide (cT20-T21, T20-T21) and a free thiol group (R-S⁻, Table I). R-S⁻, is the reactive thiolate form of the thiol and R-S-S-R its oxidized form. The dotted line represents a trypsin cleavage site in hGH.

Table I

Amino acid sequence of model peptides used to investigate thiol-disulfide exchange reactions and mixed disulfide-linked peptides from hGH for the reaction of hGH with GSH and T20a.

Peptide	Description	Amino acid sequence	Theoretical mass (Da)	Observed mass (Da)	Retention time (min)
<i>Disulfide-linked peptides</i>					
cT20-T21	Cyclic peptide	NH ₂ -IVQCRSVEGSCGF-OH	1381.6120	1381.6066	4.89
T20-T21 _{pep}	Heterodimer, disulfide-linked peptide	NH ₂ -IVQCR-OH NH ₂ -SVEGSCGF-OH	1399.6225	1399.6243	12.15
<i>Free thiol-containing peptides</i>					
rT20-T21	Monomer, free thiol group	NH ₂ -IVQCRSVEGSCGF-OH	1383.6280	1383.6176	5.10
T20a	Monomer, free thiol group	NH ₂ -GIVQCR-OH	674.3534	674.3509	8.79
T20	Monomer, free thiol group	NH ₂ -IVQCR-OH	617.3319	617.3300	5.36
GSH	Monomer, free thiol group	NH ₂ -ECG-OH	307.0838	NA	NA
RCR	Monomer, free thiol group	NH ₂ -RCR-OH	433.2220	NA	NA
QCR	Monomer, free thiol group	NH ₂ -QCR-OH	405.1794	NA	NA
CR	Monomer, free thiol group	NH ₂ -CR-OH	277.1209	NA	NA
<i>Peptides from Trypsin digestion</i>					
T20-S-S-G	Linear peptide, disulfide linked to GSH	NH ₂ -IVQCR-OH NH ₂ -ECG-OH	922.4001	922.3976	5.26
T21-S-S-G	Linear peptide, disulfide linked to GSH	NH ₂ -SVEGSCGF-OH NH ₂ -ECG-OH	1089.374	1089.367	11.99

Peptide	Description	Amino acid sequence	Theoretical mass (Da)	Observed mass (Da)	Retention time (min)
T6-S-S-G	Linear peptide, disulfide linked to GSH	NH ₂ -YSFLQNPQTSLCFSESIPTPSNR-OH NH ₂ -ECG-OH	2920.301	2920.3053	21.09
T16-S-S-G	Linear peptide, disulfide linked to GSH	NH ₂ -NYGLLYCFR-OH NH ₂ -ECG-OH	1452.617	1452.6066	19.02
T20a-T20	Scrambled disulfide-linked peptide	NH ₂ -GIVQCR-OH NH ₂ -IVQCR-OH	1289.670	1289.6604	10.89
T20a-T21	Scrambled disulfide-linked peptide	NH ₂ -GIVQCR-OH NH ₂ -SVEGSCGF-OH	1456.644	1456.6329	13.99
T20a-T6	Scrambled disulfide-linked peptide	NH ₂ -GIVQCR-OH NH ₂ -YSFLQNPQTSLCFSESIPTPSNR-OH	3287.570	3287.5724	20.32
T20a-T16	Scrambled disulfide-linked peptide	NH ₂ -GIVQCR-OH NH ₂ -NYGLLYCFR-OH	1819.886	1819.8764	17.92

Retention times were obtained on the LC-MS (See methods).

Table II

Percent decrease in concentration of the C-terminal native disulfide in hGH (T20-T21) and in a cyclic model peptide (cT20-T21) after 1 hour and 1 day of reaction with different free thiols, pH 7.0, 37 °C; n = 3.

Disulfide	Thiol	%Decrease in Native Disulfide	
		After 60 min	After 1 day
T20-T21 in hGH (intact)	GIVQCR (T20a)	38 ± 7	37 ± 6
	GSH	58 ± 6	24 ± 4
	RCR	36 ± 8	11 ± 1
	CR	21 ± 6	7 ± 2
	C	16.7 ± 0.5	4 ± 3
cT20-T21 peptide	GIVQCR (T20a)	62 ± 5	54 ± 2
	IVQCR (T20)	70 ± 1	64 ± 1
	GSH	85 ± 4	77 ± 1
	RCR	49.9 ± 0.1	23.9 ± 0.1
	QCR	59 ± 1	39.3 ± 0.5
	CR	66 ± 1	8.3 ± 0.5
	C	57.3 ± 0.4	13.7 ± 0.2