Cystine Knot Mutations Affect the Folding of the Glycoprotein Hormone α-Subunit

DIFFERENTIAL SECRETION AND ASSEMBLY OF PARTIALLY FOLDED INTERMEDIATES*

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The common glycoprotein hormone α-subunit (GPH-α) contains five intramolecular disulfide bonds, three of which form a cystine knot motif (10–60, 28–82, and 32–84). By converting each pair of cysteine residues of a given disulfide bond to alanine, we have studied the role of individual disulfide bonds in GPH-α folding and have related folding ability to secretion and assembly with the human chorionic gonadotropin β-subunit (hCG-β). Mutation of any non-cystine knot disulfide bond 7–31, bond 59–87, or both (leaving only the cystine knot) resulted in an efficiently secreted folding form that was indistinguishable from wild type. Conversely, the cystine knot mutants were inefficiently secreted (<25%). Furthermore, mutation of the cystine knot disulfide bonds resulted in multiple folding intermediates containing 1, 2, or 4 disulfide bonds. High performance liquid chromatographic separation of intracellular and secreted forms of the folding intermediates demonstrated that the most folded forms were preferentially secreted and combined with hCG-β. From these studies we conclude that: (i) the cystine knot of GPH-α is necessary and sufficient for folding and (ii) there is a direct correlation between the extent of GPH-α folding, its ability to be secreted, and its ability to heterodimerize with hCG-β.

The glycoprotein hormone α subunit (GPH-α) combines with four distinct β subunits giving rise to four biologically active hormones: chorionic gonadotropin, luteinizing hormone, thyroid-stimulating hormone, and follicle-stimulating hormone. The common glycoprotein hormone β-subunit (hCG-β) contains five intramolecular disulfide bonds, (6–10). The role of individual disulfide bonds in hCG-β secretion (11, 12) and combination with GPH-α (6, 12) has also been determined. Furthermore, site-directed mutagenesis of hCG-β has shown that the removal of disulfide bonds involved in discrete steps of the hCG-β folding pathway either disrupts the folding and assembly of hCG-β or slows progression to subsequent folding intermediates (10). Previous studies have shown that the three cystine knot disulfide bonds of GPH-α are necessary for efficient secretion and heterodimerization with hCG-β (13, 14). However, little is known about the role of individual disulfide bonds as GPH-α folds to its native conformation and how this folding process relates to GPH-α secretion and heterodimer formation.

In this study, we have determined the role of disulfide bond formation in the intracellular folding of GPH-α and have determined the relationship between the extent of folding, the ability to be secreted, and the ability to form a dimer with hCG-β. Previous attempts to study the intracellular folding of GPH-α have been unsuccessful because GPH-α folds relatively fast ($t_{1/2} = -90$ s), making the isolation of folding intermediates difficult. Therefore, to characterize the role of each disulfide bond, we have converted each pair of cysteine residues involved in a disulfide bond to alanine. From these experiments, we have determined that removal of any of the three cystine knot disulfide bonds (10–60, 28–82, or 32–84) significantly disrupted folding and resulted in multiple folding intermediates. Furthermore, we have demonstrated that the most highly folded intermediates of the cystine knot mutants are preferen-
selection in Ham's F-12 (Life Technologies, Inc.) containing G418. Plasmid DNA was also used for transient transfection into 293T cells as described below. The abbreviations used to describe the disulfide bond mutations in which both cysteine residues of a given disulfide bond were converted to alanine are: C7–31A, C10–90A, C28–82A, C32–84A, and C59–87A. The mutant missing the disulfide bonds 7–31 and 59–87 is designated as C7–31A/C59–87A.

**Transient Transfection of 293T Cells**—293T cells were plated into 60-mm plastic dishes and grown to 70–80% confluency. 5.0 ml of fresh medium was added to each dish 1 h before transfection. To precipitate the plasmid DNA, 15–20 μg of pcDNA3-α (wild type (WT) or mutant construct) was diluted to 225 μl with serum-free medium lacking cysteine (10). For experiments using dithiothreitol (DTT) during the pulse, DTT was added with the [35S]cysteine at a final concentration of 2.0 mM. Pulse incubations were carried out as described previously (9), and the cells were incubated for the chase times indicated. For secretion studies, the chase medium was saved. Cells were harvested by rinsing with cold phosphate-buffered saline (PBS) and immediately lysed in 2.5 ml of PBS containing detergents (1.0% N-lauroyl sarcosine and 0.5% sodium deoxycholate) followed by centrifugation. The supernatant was centrifuged for 1 hour at 100,000 g at 4 °C and stored at -20 °C for further experiments. The supernatant was transferred to a 15-ml conical tube for immunoprecipitation. For secretion studies, the chase medium was also clarified by centrifugation. The supernatant was transferred to a 15-ml conical tube for immunoprecipitation. Samples were stored at 4 °C until further analysis.

**Metabolic Labeling with [35S]Cysteine**—Transfected 293T or Chinese hamster ovary cells were pulse-labeled for the times indicated in the text with [35S]Cysteine (~1100 Ci/mmol; NEN Life Science Products), at a concentration of 100–150 μCi/ml, in serum-free medium lacking cysteine (10). For experiments using dithiothreitol (DTT) during the pulse, DTT was added with the [35S]cysteine at a final concentration of 2.0 mM. Pulse incubations were carried out as described previously (9), and the cells were incubated for the chase times indicated. For secretion studies, the chase medium was saved. Cells were harvested by rinsing with cold phosphate-buffered saline (PBS) and immediately lysed in 2.5 ml of PBS containing detergents (1.0% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS); protease inhibitors (20 mM EDTA and 2 mM phenylmethanesulfonyl fluoride); and 5 mM N-ethylmaleimide (NEM), pH 7.0, to trap free thiol groups of the GPH-α folding intermediates that contained unformed disulfide bonds. Cell lysates were incubated 10–20 min at room temperature in the dark, followed by disruption through a 22-gauge needle (five times) and centrifuged for 1 h at 100,000 g at 4 °C. For secretion studies, the chase medium was also clarified by centrifugation. The supernatant was transferred to a 15-ml conical tube for immunoprecipitation. For secretion studies, the chase medium was also clarified by centrifugation. The supernatant was transferred to a 15-ml conical tube for immunoprecipitation. Samples were stored at 4 °C until further analysis.

**Immunoprecipitation of Cell Lysates and Culture Media**—Immunoprecipitation was performed using the manufacturer’s protocol. Stable Chinese hamster ovary cell lines were made by calcium phosphate precipitation of plasmid DNA followed by partially secreted and combine with hCG-β, whereas the less folded forms are more highly degraded. Removal of each non-cystine knot disulfide bond individually or in combination, however, did not affect folding or secretion. To our knowledge, the results reported here are the first to describe the construction and folding of a cystine knot-containing protein from which all other disulfide bonds have been removed, except those involved in the knot. This has enabled us to specifically study the role of the cystine knot in GPH-α secretion and heterodimerization with hCG-β and should serve as a simplified model protein for future studies involving the cystine knot motif.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293T cells (15) were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum, and penicillin (100 units/ml)/streptomycin (100 μg/ml) (Life Technologies, Inc.). Chinese hamster ovary cells were grown in Ham’s F-12 (Life Technologies, Inc.) as described previously (10).

**Site-directed Mutagenesis and Expression of Recombinant GPH-α—** Cysteine to alanine mutations were made using “megaprimer” polymerase chain reaction (16) with Pyr polymerase (Stratagene). The 410-bp pair fragment was cloned into pcDNA3 (Invitrogen) using HindIII and ApaI restriction sites. Clones containing an insert were sequenced to confirm the presence of the desired mutation(s). Plasmid preparation was performed using the Maxi Plasmid Kit (Qiagen) following the manufacturer’s protocol. Stable Chinese hamster ovary cell lines were made by calcium phosphate precipitation of plasmid DNA followed by

**SDS-PAGE and Autoradiography**—Radiolabeled GPH-α forms that

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**FIG. 1. Schematic diagram of GPH-α.** The crystal structure (1) of GPH-α indicates that five disulfide bonds are formed in the native structure: 7–31, 10–60, 28–82, 32–84, and 59–87. The cysteine residues displayed as filled circles form the cystine knot; disulfide bonds 28–82 and 32–84 form a ring structure through which disulfide bond 7–31 penetrates. Disulfide bonds 7–31 and 59–87 (open circles) are non-cystine knot disulfide bonds. The topology of GPH-α consists of three loops that are stabilized by the disulfide bonds. The three loops are labeled as L1, L2, and L3, respectively.
adenosorbed to protein-A-Sepharose beads were prepared as described previously (9). Briefly, GPH-α was eluted with twice concentrated SDS gel sample buffer (125 mM Tris-HCl, pH 6.8, containing 2% SDS, 20% glycerol, and 40 μg/ml bromphenol blue). The samples, including the protein A-Sepharose beads, were boiled for 5 min, loaded on polyacrylamide gradient slab gels (5–20%), and run by the method of Laemmli (17). The gels were dried in vacuo on filter paper and exposed to a phosphor screen (Molecular Dynamics). The exposed phosphor screen was scanned on the Molecular Dynamics Storm 860 using Molecular Dynamics Scanner Control (version 4.1) software. Bands were quantitated using the Molecular Dynamics ImageQuant (version 4.2a) volume report.

Amino Acid Analysis Procedure to Determine Disulfide Bond Content—A modified protocol, similar to the one used in determining the disulfide folding pathways for potato carboxypeptidase inhibitor and human epidermal growth factor (18, 19) was used. [35S]Cysteine containing folding forms of GPH-α isolated from reversed-phase HPLC were dried in vacuo and hydrolyzed by vapor phase HCl under vacuum for 75 min at 150 °C in a Waters Pico-Tag work station. Quantitation of [35S]Cysteine and succinyl-[35S]Cysteine (hydridosolysis product of NEM-Cys) was performed using a modification of the method described by Cohen and Michaud (20). Hydrolysates were resuspended in 10 μl of 10 mM HCl. To this, 70 μl of 0.2 n barote buffer, pH 8.8, was added. Derivatization of amino acids was performed by adding 30 μl of 6-aminoquinoly-N-hydroxysuccinimidyl carbamate (8 mg/ml in anhydrous acetoni-

trile). Samples were dried in 110 μl of buffer A (140 mM sodium acetate, 17 mM triethylyamine, pH 4.9). Separation of derivatized amino acids was performed using a Waters Nova-Pack 4 μm C18 column using buffer A and buffer B (60% acetonitrile in water). The column was eluted using the following gradient: (i) 3% B to 11% B from 0—20 min, (ii) 11% B to 37% B from 20—45 min, (iii) 37% B to 100% B from 45—48 min, and (iv) reequilibration at 0% B for 15 min. The column was eluted at 1.0 ml/min at 30 °C, and 1-ml fractions were collected and quantitated by scintillation counting. The peak containing [35S]Cysteine represented cysteine residues involved in a disulfide bond, whereas succinyl-[35S]Cysteine represented unfolded disulfide bonds. The percentage of [35S]Cysteine and succinyl-[35S]Cysteine was calculated by dividing the cpm recovered for each fraction by the total cpm recovered with the total cpm recovered for each fraction of the Sepharose beads prior to HPLC dissociates the α/β heterodimer (10). Although the dimer was dissociated, the GPH-α and hCG-β subunits co-eluted and therefore were not resolved by reversed-phase HPLC. Fractions containing [35S]Cysteine-labeled GPH-α and hCG-β were combined and concentrated in vacuo, and the volume and pH was adjusted using PBS containing detergents. To separate the dissociated subunits, GPH-α was immunoprecipitated with GPH-α polyclonal anti-

serum. This step isolated the forms of GPH-α that co-precipitated with hCG-β and, thus, represents assembly-competent GPH-α folding forms. Following immunoprecipitation, a second immunoprecipitation was performed with hCG-β polyclonal antisera. The immunoprecipi-

tated GPH-α and hCG-β were each analyzed by reversed-phase HPLC as described above.

RESULTS

The Effect of DTT on GPH-α Folding—The intracellular kinetics of GPH-α folding is relatively fast (t1/2 ≈ 90 s), making the analysis of short time points (0—5 min) in pulse-chase experiments difficult. This is because most of the [35S]Cysteine-labeled GPH-α has achieved a fully folded conformation during a 3–5-

min pulse. To more effectively study GPH-α folding, we needed a strategy to better control the kinetics of GPH-α folding. Treatment of cells with DTT reversibly retains proteins in the endoplasmic reticulum that require disulfide bond formation for folding, while having no detectable effect on secretory proteins that do not contain disulfide bonds (21). We hypothesized that if DTT treatment reversibly blocked the folding of GPH-α, we could use DTT during the pulse phase to allow [35S]Cysteine labeling while maintaining GPH-α in its unfolded confor-

mation. Subsequent folding steps could then be defined after re-

moval of DTT. To examine this, 293T cells transiently expressing GPH-α were radiolabeled with [35S]Cysteine for 10 min in the presence of 2 mM DTT followed by the chase conditions indicated. A, reversed-phase HPLC profile of GPH-α after no chase, indicating that the elution position of the unfolded subunit (αu) was at 50 min. The αu species was isolated, and the percentage of [35S]Cysteine (S-S) and NEM-alkylated [35S]Cysteine (Cys) was determined as de-

scribed under “Experimental Procedures” and is shown in the bar graph to the right of each panel. >95% of the [35S] cpm were recovered as alkylated cysteine, indicating that no disulfide bonds had formed. B, 5-min chase in the presence of 2 mM DTT, indicating that GPH-α had remained in its unfolded state containing no disulfide bonds. C, 5-min chase in the absence of DTT, demonstrating the conversion from αu to the native conformation (αN) that eluted at 28 min. Recovery of >98% of [35S] as cysteine for αN confirmed that all five disulfide bonds were formed. The S-S content shown in each bar graph is the average of at least three independent experiments.

![Image](http://www.jbc.org/Downloadedfrom)
that combined with hCG-β (10), and that eluted at 28 min on reversed-phase HPLC. The folding from the unfolded species (αu) (Fig. 2, A and B) to the less hydrophobic, native conformation (αN) (Fig. 2C) resulted in a shift to an earlier elution time (28 min). Thus, treatment of 293T cells with 2 mM DTT inhibited the folding of GPH-α, and this inhibition was reversible when DTT was removed.

The Role of Individual Disulfide Bonds in GPH-α Folding—Previous studies have shown that mutation of the cystine knot disulfide bonds (10–60, 28–82, and 32–84) results in an unstable molecule that is highly degraded and combines inefficiently with hCG-β (13, 14). Based on these observations, we hypothesized that formation of the cystine knot plays an important structural role in the ability of GPH-α to fold into its native conformation. To test this hypothesis, five mutants were created by converting each pair of cysteine residues to alanine such that each mutant lacked the ability to form one of the five disulfide bonds. The mutants created were C7–31A, C59–87A, C10–60A, C28–82A, and C32–84A (see Fig. 1 for disulfide bond locations).

Disulfide bonds 7–31 and 59–87 of GPH-α are located in loops 1 and 3, respectively, and are not involved in the cystine knot motif. Pulse-chase studies were performed to determine the ability of the two non-cystine knot mutants, C7–31A and C59–87A, to fold. 293T cells transiently expressing C7–31A or C59–87A were radiolabeled with [35S]cysteine for 10 min in the presence of 2 mM DTT followed by no chase (0 min) or a 5 min chase in the absence of DTT to allow folding to proceed. Immunoprecipitated folding forms were separated using reversed-phase HPLC. The unfolded form of each mutant (αu) is shown in the panels labeled 0 min. The vertical dotted line represents the elution position of native WT-α and indicates that the folded forms (αf) of C7–31A, C59–87A, and C7–31A/C59–87A all attained a native-like conformation that eluted at the same position as WT-α.

Fig. 3. HPLC analysis of non-cystine knot mutants. 293T cells transiently transfected with pcDNA3-α containing the C7–31A mutation, the C59–87A mutation, or both C7–31A and C59–87A mutations (C7–31A/C59–87A) were radiolabeled with [35S]cysteine for 10 min in the presence of 2 mM DTT followed by no chase (0 min) or a 5 min chase in the absence of DTT to allow folding to occur. Immunoprecipitated folding forms were separated using reversed-phase HPLC. The unfolded form of each mutant (αu) is shown in the panels labeled 0 min. The vertical dotted line represents the elution position of native WT-α and is located at an earlier elution position relative to all folding forms isolated for each cystine knot mutant. Because the elution time for the most folded form of each mutant (C10–60A α3, C28–82A α4, and C32–84A α3) was later than native WT-α, none of these forms were classified as being fully folded (αf). Beginning with the least folded form for each mutant the peaks were labeled α1, α2, etc. The α1 form for each mutant eluted close to αu; however, the elution time of α1 was about 1–4 min earlier. It should noted that a given peak for a particular mutant does not necessarily represent the same folding form for the other two mutants (i.e. C10–60A α3 is not necessarily the same as C28–82A α4 and C32–84A α3).

Fig. 4. HPLC analysis of intermediates isolated from GPH-α cystine knot mutants. 293T cells transiently transfected with pcDNA3-α C10–60A, C28–82A, or C32–84A were radiolabeled with [35S]cysteine for 10 min in the presence of 2 mM DTT followed by chase times of 0, 2, or 10 min in the absence of DTT to allow folding to proceed. Immunoprecipitated folding forms were separated using reversed-phase HPLC. The unfolded form of each mutant (αu) is shown in the panels labeled 0 min. The vertical dotted line represents the elution position of native WT-α and is located at an earlier elution position relative to all folding forms isolated for each cystine knot mutant. Because the elution time for the most folded form of each mutant (C10–60A α3, C28–82A α4, and C32–84A α3) was later than native WT-α, none of these forms were classified as being fully folded (αf).
because HPLC elution times may not necessarily separate all native and native-like species. Based on these results, we next wanted to determine whether the three cystine knot disulfide bonds were sufficient for folding. To address this issue, a mutant lacking both disulfide bonds 7–31 and 59–87 (C7–31A/C59–87A) was constructed and analyzed as described above. The simultaneous mutation of disulfide bonds 7–31 and 59–87 resulted in a protein that contained only the three cystine knot disulfide bonds. As shown in Fig. 3, the C7–31A/C59–87A mutant folded within 5 min to a form indistinguishable from that of the C7–31A and C59–87A single disulfide mutants and that of native WT-α. These data demonstrate that the non-cystine knot disulfide bonds 7–31 and 59–87 are not required for folding to a form that is indistinguishable from WT GPH-α. Thus, the three cystine knot disulfide bonds appear to be sufficient for folding and may function as the major structural framework for GPH-α.

In contrast to the C7–31A and C59–87A mutants, disruption of any of the cystine knot disulfide bonds (10–60, 28–82, or 32–84) resulted in multiple folding forms (Fig. 4). Using HPLC elution times as an indicator of relative hydrophobicity, the degree of folding of the various intermediates for each mutant was assessed. Thus, within each series of intermediates, α1 was the least folded, and α5 or α6 was the most folded. Based on this analysis, none of these intermediates were as folded as native WT-α. As shown in Fig. 4, the multiple folding intermediates were present at 2 min and remained for up to 10 min of chase. Chase times of greater than 10 min did not result in further progression of the less folded forms to the most folded form (data not shown), suggesting that a portion of the less folded forms became trapped. Studies on the in vitro folding of bovine pancreatic trypsin inhibitor have established precedence for kinetically trapped, dead-end intermediates (22, 23). To ensure that the isolation of multiple folding forms was not due to labeling in the presence of 2 mM DTT, experiments were performed without DTT, and the same profile of intermediates was obtained (data not shown). Furthermore, Chinese hamster ovary cells stably expressing each of these mutants gave similar results, suggesting that the folding observed was not cell type-specific (data not shown). Taken together, the analysis of folding intermediates for each disulfide bond mutant supports our hypothesis that the cystine knot disulfide bonds play a more important role in attaining a native conformation than the two non-cystine knot disulfide bonds.

Quantitation of Disulfide Bond Formation in GPH-α Variants—The data presented in the previous section demonstrated the importance of the three cystine knot disulfide bonds. Their disruption by mutagenesis resulted in multiple intermediates representing non-native conformations. To further investigate the nature of the folding forms isolated for the disulfide bond mutants, we determined the number of disulfide bonds formed for each of the isolated species. Fractions representing the folding forms observed in Figs. 3 and 4 were isolated by reversed-phase HPLC and further analyzed by amino acid analysis. As described under “Experimental Procedures,” acid hydrolysis of the isolated folding forms resulted in the production of [35S]succinyl-cysteine (i.e. hydrolysis product of [35S]NEM-cysteine), [35S]cysteine (i.e. disulfide-linked cysteine residues), and other unlabeled amino acids. After precolumn derivatization, separation of amino acids was performed using C18 reversed-phase HPLC. The percentage of disulfide bond formation and the number of disulfide bonds formed was determined by quantitation of [35S]cysteine and [35S]succinyl-cysteine and is shown in Table I. As shown in Fig. 2 and Table I, folded WT-α contained all five disulfide bonds. In addition, analysis of the non-cystine knot mutants lacking disulfide bonds 7–31 or 59–87 contained four formed disulfide bonds, whereas the mutant lacking both the 7–31 and 59–87 disulfide bonds contained three formed disulfide bonds. These data provide further support for the hypothesis that the cystine knot is the major determinant in GPH-α folding and that the non-cystine knot disulfide bonds are not necessary for folding or the formation of the cystine knot.

The most folded form isolated from the C10–60A mutant, designated as α3 (Fig. 4, 10 min chase), contained about 98% of the [35S] counts in the cysteine form, indicating that this species also contained four disulfide bonds. Likewise, the most folded forms of the other two cystine knot mutants, C28–82A α4 and C32–84A α5, also contained four intact disulfide bonds. The fact that all four remaining disulfide bonds can form in these mutants, albeit by an inefficient process, suggests that no disulfide bonds are required to form before subsequent disulfide bonds can form in the folding pathway. However, we cannot rule out the possibility that some non-native disulfide bonds may be present. C28–82A and C32–84A mutations, which disrupt the formation of the cystine knot ring, resulted in the accumulation of four and three intermediates, respectively. As shown in Table I, C28–82A α4 contained two disulfide bonds, α5 contained one or two disulfide bonds, and α6 contained one disulfide bond. Similarly, C32–84A α5 contained two disulfide bonds and α6 contained one disulfide bond, indicating a direct correlation between the extent of folding and disulfide bond formation. As mentioned above, these less folded forms failed to convert efficiently to the most folded form.

### Table I

| GPH-α variant | S-S | NEM-Cys | Number of S-S bonds |
|---------------|-----|---------|---------------------|
| α-WT          | 98.7 ± 0.9 | 1.2 ± 0.7 | 4.94 (5) |
| α-C7–31A      | 98.0 ± 1.7 | 2.1 ± 2.0 | 3.92 (4) |
| α-C59-C87A    | 98.8 ± 1.6 | 1.3 ± 1.7 | 3.95 (4) |
| α-C7–31A/C59–87A | 99.3 ± 0.5 | 0.7 ± 0.5 | 2.98 (3) |
| α-C10–60A     | 10.3 ± 7.1 | 89.7 ± 7.0 | 0.41 (4) |
| α-C28–82A     | 32.0 ± 8.5 | 68.0 ± 8.5 | 1.28 (4) |
| α-C32–84A     | 98.1 ± 1.5 | 1.9 ± 1.4 | 3.92 (4) |
| αα1           | 23.3 ± 8.0 | 76.8 ± 8.0 | 0.95 (4) |
| αα5           | 34.7 ± 5.9 | 65.3 ± 5.9 | 1.39 (4) |
| αα6           | 55.7 ± 11.7 | 44.3 ± 11.7 | 2.23 (4) |
| αα7           | 96.0 ± 2.9 | 3.9 ± 2.8 | 3.84 (4) |
| αα8           | 31.7 ± 5.5 | 68.3 ± 5.5 | 1.27 (4) |
| αα9           | 56.8 ± 8.1 | 43.2 ± 8.1 | 2.27 (4) |
| αα10          | 98.3 ± 1.9 | 1.6 ± 1.9 | 3.93 (4) |

* Average of at least three independent experiments ± S.D.
* Calculated as percentage S-S × total possible disulfide bonds that could theoretically form. The number in parentheses denotes the total possible disulfide bonds for each GPH-α variant.
indicating that some of these species appear to have become trapped.

**The Effect of Disulfide Bond Mutations on GPH-α Secretion**—The ability of a secretory protein to fold to its native conformation in the endoplasmic reticulum is generally required to prevent degradation by the quality control system that recognizes unfolded conformations (24, 25). Because the non-cystine knot mutants C7–31A and C59–87A appear to fold to a native-like conformation (Fig. 3) containing all other disulfide bonds (Table I) and because the cystine knot mutants C10–60A, C28–82A, and C32–84A could not fold to a native-like conformation (Fig. 4), we asked the following question: does the extent of GPH-α folding correlate with the ability to be secreted? To address this question, 293T cells transiently expressing WT-α or the disulfide bond mutants were pulse-labeled for 10 min with [35S]cysteine and chased for 10 min, 1 h, 2 h, 4 h, or 8 h. The immunopurified cell lysates and medium containing GPH-α were analyzed by SDS-PAGE and quantitated by autoradiography. About 75% of the [35S]cysteine-labeled WT-α present at 10 min was recovered in the medium at 8 h with <10% remaining in the cell (Fig. 5A). Similar to WT-α, the C7–31A, C59–87A, and C7–31A/C59–87A mutants were also efficiently secreted (>70%). Therefore, the ability of the non-cystine knot disulfide mutants to fold to a native-like form that elutes at the same position as WT-α on reversed-phase HPLC (Fig. 3) correlates with their ability to be efficiently secreted. In contrast, only about 20–25% of each cystine knot mutant (C10–60A, C28–82A, and C32–84A) was secreted, whereas the majority was degraded intracellularly within 4 h (Fig. 5B). This demonstrates that an intact cystine knot is critical for GPH-α to attain a structure that allows it to be efficiently secreted.

**Differential Secretion and Combination of C28–82A and C32–84A Intermediates**—The SDS-PAGE analysis of cystine knot mutants (Fig. 5) clearly showed that disruption of the knot structure resulted in inefficient secretion; however, it did not distinguish between the fates of the different intermediates. The isolation of multiple intermediates, some being more folded than others, provided a novel system to address the following question: do the different folding intermediates contain differing abilities to translocate through the endoplasmic reticulum and be secreted? To address this question, 293T cells transiently expressing C28–82A or C32–84A were pulse-labeled for 10 min with [35S]cysteine and chased for 10 min or 2 h. Because the majority of C10–60A folds to a single form, C10–60A α4, this mutant was not analyzed. A 2-h chase time was chosen so that a sufficient amount of intracellular [35S]cysteine-labeled GPH-α remained for analysis. The immunopurified cell lysates (10 min and 2 h) and medium (2 h) were analyzed by reversed-phase HPLC to determine the amount of each intermediate secreted into the medium and the amount remaining intracellularly (Fig. 6, A and B). The 2-h chase (secreted) HPLC profile contained mainly the most folded form of each mutant (C28–82A α4 and C32–84A α5). The percentage
of each intermediate remaining after 2 h, either intracellular or secreted, is shown in Fig. 6 (C and D). For example, of the amount of C28–82A $\alpha_1$ remaining at 2 h (42% of the original labeled material seen at 10 min), half was retained in the cell, and half was secreted, whereas for C28–82A $\alpha_1 < 5$% was secreted. These data show that the most folded forms were more efficiently secreted than the less folded forms. Furthermore, about 40–45% of the total (cellular + secreted) C28–82A $\alpha_4$ and C32–84A $\alpha_3$ remained after 2 h, whereas only 25–30% of the less folded forms remained, indicating that the most folded intermediates were less susceptible to degradation. These results demonstrate that the ability of different intermediates to be secreted also correlates with the overall extent of folding.

A previous report by Sato et al. (14) showed that C28–82A and C32–84A combined inefficiently with hCG-β. We have made similar observations in our laboratory (data not shown). Based on our data demonstrating that C28–82A $\alpha_4$ and C32–84A $\alpha_3$ each contained four disulfide bonds and were preferentially secreted, we hypothesized that they may also be the only intermediates that could combine with hCG-β. To test this, 293T cells transiently expressing both hCG-β and C28–82A or C32–84A were pulse-labeled for 30 min with $[^{35}S]$cysteine and chased for 6 h. The collected medium was immunoprecipitated first with a polyclonal antibody to hCG-β. Because this antibody recognizes assembled and unassembled hCG-β, it also co-immunoprecipitated the forms of the each GPH-α mutant that were assembled with hCG-β. After the assembled forms of each GPH-α mutant were co-precipitated with hCG-β, the heterodimer was dissociated as described under “Experimental Methods.” The GPH-α forms released from the heterodimer represented those that were assembly-competent. Next, the assembly-competent forms were immunopurified with polyclonal α-antiserum and analyzed by reversed-phase HPLC. As shown in Fig. 7 (A and D), C28–82A $\alpha_4$ and C32–84A $\alpha_3$ were the only forms of each respective mutant that was secreted as a heterodimer with hCG-β. C28–82A $\alpha_1$ and C32–84A $\alpha_2$ represent the most folded form of each mutant (Fig. 4). After immunoprecipitating with polyclonal α-antiserum, a second immunoprecipitation was performed using polyclonal hCG-β antiserum. The precipitated hCG-β was also analyzed by reversed-phase HPLC (Fig. 7, B and E). Because hCG-β and GPH-α eluted from the HPLC at similar positions (Fig. 7, compare A with B and D with E), we confirmed that each peak in Fig. 7 (A and D) was actually GPH-α by its migration on SDS-PAGE (Fig. 7, C, lane 1, and F, lane 1). Similarly, the peaks that were observed in Fig. 7 (B and E) represented only hCG-β (Fig. 7, C, lane 2, and F, lane 2). A control experiment was performed to assess whether or not coexpression of hCG-β with C28–82A and C32–84A GPH-α altered the profile of intracellular folded intermediates. Results obtained indicate that the folding forms observed for C28–82A and C32–84A were the same in the absence or presence of hCG-β (data not shown). These data indicate that the most folded forms of C28–82A and C32–84A were assembly-competent despite being incompletely folded. Furthermore, these data imply that the extent of conversion to C28–82A $\alpha_4$ and C32–84A $\alpha_3$ is what ultimately determines the efficiency of secretion and combination for these two mutants.

**DISCUSSION**

GPH-α is a unique subunit in that it contains intrinsic structural characteristics that allow it to combine with four different β subunits, suggesting that GPH-α must contain a high degree of flexibility. Indeed, recent NMR studies (26, 27) on the solution structure of the free (uncombined) GPH-α subunit reveal a group of 27 structures, an extremely disordered loop 2, and well defined loops 1 and 3 (Fig. 1). The core structural feature of GPH-α that stabilizes this otherwise flexible molecule is five disulfide bonds that function as a molecular scaffold. In this study, we have used site-directed mutagenesis to investigate the importance of these five disulfide bonds in GPH-α folding and secretion. Additionally, the recovery of multiple intracellular folding intermediates that did not progress to one final conformation allowed for a unique investigation into the relationship between the extent of folding and the ability of these different intermediates to be secreted and combine with hCG-β.

The data from C7–31A and C59–87A mutations demonstrated that GPH-α lacking either disulfide bond 7–31 or disulfide bond 59–87 was capable of folding to a native-like conformation in which the remaining four disulfide bonds were intact. Additionally, a mutant lacking both the 7–31 and 59–87 disulfide bonds attained a conformation that contained three of three possible disulfide bonds and whose folding and secretion were indistinguishable from the C7–31A and C59–87A single
DISULFIDE MUTANTS

GPH-α Disulfide Mutants

Fig. 7. Assembly-competent forms of C28-82A and C32-84A intermediates. 293T cells transiently transfected with both pcDNA3-α (C28-82A or C32-84A) and pcDNA3-hCG-β were radiolabeled with [35S]cysteine for 30 min and chased for 6 h. The medium was collected and immunoprecipitated with a polyclonal hCG-β antibody that recognizes both uncombined and combined hCG-β and thus, co-precipitated assembly-competent forms of GPH-α. After isolation and subsequent dissociation of the immunoprecipitated α/β heterodimer (see “Experimental Procedures”), a polyclonal GPH-α antibody was used to isolate the form(s) of GPH-α that had combined with hCG-β. A and D represent the HPLC profiles of the assembly-competent forms of C28-82A α and C32-84A α, respectively. After immunoprecipitation with the GPH-α antibody, a second immunoprecipitation was performed using the hCG-β antibody. B and E represent the HPLC profile of mature, secreted hCG-β. Because the GPH-α and hCG-β eluted at similar positions on HPLC, we confirmed that the peaks isolated were GPH-α and hCG-β by nonreducing SDS-PAGE. C represents SDS-PAGE analysis of the HPLC peaks immunopurified with the GPH-α (lane 1) and hCG-β (lane 2) antibodies from A and B, respectively. F represents SDS-PAGE analysis of the HPLC peaks immunopurified with the GPH-α (lane 1) and hCG-β (lane 2) antibodies from D and E, respectively. The migration of GPH-α (22 kDa) and hCG-β (35 kDa) confirmed that the peak isolated with each respective antibody was only GPH-α or hCG-β. Molecular weight markers (M) are (from top): ovalbumin (Mr = 45,000) and carbonic anhydrase (Mr = 29,000).

mutants and WT. It should be noted that the mutant lacking both disulfide bonds 7–31 and 59–87 contains only the three disulfide bonds that define the cystine knot. Because this mutant contains three disulfide bonds, folds to a form indistinguishable from WT based on HPLC elution position, and is secreted as efficiently as WT, we conclude that the cystine knot is intact. This suggests that the 7–31 and 59–87 disulfide bonds are not critical for folding or the formation of the cystine knot and that the cystine knot is sufficient for efficient folding and secretion.

Disulfide bonds 7–31 and 59–87 are, however, presumably important for other aspects of biological function, such as heterodimer formation or receptor binding, for one or more of the four glycoprotein hormones (chorionic gonadotropin, luteinizing hormone, thyroid-stimulating hormone, and follicle-stimulating hormone). The importance of these two bonds is implied by the fact that they are highly conserved across species (28). Although biochemical studies have shown that disruption of the 7–31 or the 59–87 disulfide bond in GPH-α does not significantly affect assembly with hCG-β (13, 29), we found that when both 7–31 and 59–87 disulfide bonds were removed at the same time, <10% of the secreted material had dimerized with hCG-β (data not shown). This suggests that deletion of either disulfide bond 7–31 or 59–87 may result in subtle conformational changes that still allow for dimerization, but removal of both disulfide bonds causes a larger conformational change that prevents dimerization with hCG-β. hCG receptor binding and signal transduction are not affected by the C7–31A mutation (13). However, receptor binding is decreased about 10-fold for C59–87A, suggesting that the C-terminal portion of GPH-α may an important receptor interaction site (13). Consistent with this observation are studies where mutation of the C-terminal amino acids of GPH-α did not affect dimerization with hCG-β but significantly decreased receptor binding (30, 31).

Mutagenesis of the cystine knot disulfide bonds of GPH-α resulted in several folding intermediates, all of which eluted at a later time than WT on reversed-phase HPLC, indicating that these mutant forms had a more hydrophobic, non-native conformation. The folding patterns observed provide an explanation for previous observations that mutation of cystine knot disulfide bonds disrupts dimerization with hCG-β (13, 14). Surprisingly, the most folded forms (C10-60A α1, C28–82A α2, and C32–84A α1) all contained four of four possible disulfide bonds, making it likely that none of the disulfide bonds in GPH-α are absolutely dependent upon the prior formation of another disulfide bond. This observation is different from what was observed for hCG-β folding (10, 11), where mutation of
disulfide bonds involved in discrete steps in the folding pathway either inhibited progression to subsequent intermediates or slowed the rate of conversion. However, it must be noted that progression to the most folded conformation is inefficient for the GPH-α cystine knot mutants, especially C28–82A and C32–84A, since less folded intermediates accumulate and never reach the most folded conformation. The less folded intermediates of the GPH-α cystine knot mutants mainly consisted of 1- and 2-disulfide bond species. A potential explanation as to why these forms do not progress to the most folded form is that the less folded intermediates represent species that contain non-native disulfide bonds that are inefficiently rearranged.

Although the overall combination of GPH-α C28–82A and C32–84A with hCG-β was inefficient, we were able to show that the population that did combine with hCG-β represented only the most folded conformation of each respective mutant. These conformations were also the forms that were preferentially secreted. Because of their inefficient assembly, low yields of dimer have restricted receptor binding and signal transduction studies. To circumvent this problem, Sato et al. (14) created fusion proteins between the GPH-α cystine knot mutants and hCG-β to bypass the assembly step. Interestingly, these fusion proteins are biologically active, suggesting that the cystine knot disulfide bonds are essential for folding to an assembly-competent conformation but not for receptor binding and signal transduction (14). Our studies showing that the efficiency of folding is the limiting factor in heterodimer formation provides a biochemical explanation for these results.

The effect of disulfide bond mutations on secretion correlated with the extent of folding observed by HPLC as indicated by the ability of C7–31A, C59–87A, and the double C7–31A/C59–87A disulfide mutant to be efficiently secreted, whereas the three cystine knot mutants (C10–60A, C28–82A, and C32–84A) were inefficiently secreted. Furthermore, the most folded intermediates of C28–82A and C32–84A were preferentially secreted and combined with hCG-β, whereas the less folded forms were more highly degraded. Although the efficiency of secretion and the ability to combine with hCG-β were related for C28–82A and C32–84A intermediates, this was not true for all mutants. For example, the α-subunit containing the double C7–31A/C59–87A mutations was secreted as efficiently as WT (Fig. 5), but <10% combined with hCG-β (data not shown), suggesting that structural features necessary for secretion of uncombined GPH-α are distinct from those required for heterodimer formation.

The findings presented in this manuscript provide information regarding the importance of the GPH-α cystine knot motif. These findings are likely to be applicable to other cystine knot-containing proteins such as transforming growth factor-β, nerve growth factor, platelet-derived growth factor, and vascular endothelial growth factor. The importance of the cystine knot for biological function is demonstrated by naturally occurring mutations predicted to disrupt the cystine knot structure. For example, a thyroid-stimulating hormone deficiency results from a glycine to arginine substitution in the thyroid-stimulating hormone β-subunit. This amino acid change prevents dimerization with GPH-α, presumably because of disruption of the cystine knot since the glycine affected is invariant among related cystine knot proteins (32). Additionally, several mutations within the Norrie disease protein (33) result in congenital blindness, and these mutations are expected to disrupt its predicted cystine knot (34).

The double disulfide bond mutant of GPH-α (C7–31A/C59–87A) studied in our laboratory contains only the three cystine knot disulfide bonds and folds normally. To our knowledge, this is the first study of a protein engineered to contain only a cystine knot without its other disulfide bonds and should aid in future studies on the role of the cystine knot in structure-function relationships. Using this mutant as a model protein, we are currently studying how cystine knots form and are determining the importance of other amino acids within the cystine knot motif.

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Cystine Knot Mutations Affect the Folding of the Glycoprotein Hormone α-Subunit: DIFFERENTIAL SECRETION AND ASSEMBLY OF PARTIALLY FOLDED INTERMEDIATES

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