Domain-dependent Protein Folding Is Indicated by the Intracellular Kinetics of Disulfide Bond Formation of Human Chorionic Gonadotropin \(\beta\) Subunit

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We have measured the intracellular rates of formation of the six disulfide bonds in the human chorionic gonadotropin \(\beta\) subunit (hCG-\(\beta\)) to determine whether the folding pathway of this molecule can be described by a simple sequential model. If such a model is correct, the formation of disulfide bonds, which is indicative of tertiary structural changes during protein folding, should occur in a discrete order. The individual rates of disulfide bridging were determined by identifying the extent of disulfide bond formation in hCG-\(\beta\) intermediates purified from choriocarcinoma cells that had been metabolically labeled for 40 or 120 s and chased for 0 to 25 min. The results of these kinetic studies describe a folding pathway in which the disulfide bonds between cysteines 34–88, 38–57, 9–90 and 23–72 stabilize, in a discrete order, the putative domain(s) involving amino acids 1–90 of hCG-\(\beta\). However, the S-S bonds 93–100 and 26–110 begin to form before the complete formation of the disulfide bonds that stabilize the amino acid 1–90 domain(s), and continue to form after complete formation of these disulfide bonds, suggesting that hCG-\(\beta\) does not fold by a simple sequential pathway. The order of completion of each of the six disulfide bonds of hCG-\(\beta\) is: 34–88 (\(t_\text{s} \approx 1–2\) min), 38–57 (\(t_\text{s} \approx 2–3\) min), 9–90 and 23–72, 93–100, and 26–110. Moreover, 60–100% of each of the six disulfide bonds form posttranslationally, and nonnative disulfide bonds do not form in detectable amounts during intracellular folding of hCG-\(\beta\).

A fundamental question associated with protein folding is whether the intramolecular interactions that stabilize secondary and tertiary structures occur in a defined order. In the simplest case, these interactions could occur in a strict order (a sequential pathway), while in the most complex model, interactions would occur randomly. Several reports have shown that secondary structure forms before tertiary structure (1–3), indicating that it is unlikely that protein folding is a random process. Less is known, however, about the order in which tertiary interactions stabilize a particular protein conformation. In vitro studies of the kinetics of disulfide bond formation in bovine pancreatic trypsin inhibitor indicate that, at least for some proteins, tertiary interactions in a protein occur in a defined order (4–6). For larger proteins, multiple domains within the protein may fold independently, and then achieve a higher order of packing at a later stage of the folding process (7). However, different results have been reported for other proteins and suggest that tertiary structure formation, as indicated by disulfide bond formation, can occur by multiple pathways via multiple intermediates (8). Such flexibility in the protein folding process is consistent with the molten globule model of protein folding, which states that secondary structures form early in the sequence of events leading to a native conformation but that these secondary structures exist in a fluctuating state (9).

We have studied the kinetics of disulfide bond formation in the \(\beta\) subunit of the glycoprotein hormone human chorionic gonadotropin (hCG)1 to elucidate a pathway of tertiary structure stabilization for this protein. Unlike most of the published studies on protein folding kinetics, which have studied folding of an isolated protein in vitro, we have measured the kinetics of folding of hCG-\(\beta\) as it occurs in cultured JAR choriocarcinoma cells. This model system reflects the folding pathway of hCG-\(\beta\) that occurs in cells of the human placenta, where the hCG hormone is normally produced (10). Furthermore, the same hCG-\(\beta\) folding pathway has been identified in nonmalignant Chinese hamster ovary cells transfected with wild type hCG-\(\alpha\) and hCG-\(\beta\) genes (11). This suggests that the folding of hCG-\(\beta\) does not depend on the type of mammalian cell in which it is expressed.

hCG-\(\beta\) is composed of 145 amino acids (molecular mass, 22 kDa) and is stabilized by six intramolecular disulfide bonds. The rate-limiting event of hCG-\(\beta\) folding in JAR choriocarcinoma cells occurs with a half-time of 4 min and is associated with a change in the amount of disulfide bond formation in the molecule (12). This comparatively slow folding rate permitted the analysis of the intracellular hCG-\(\beta\) folding pathway that was described by the order of formation of the six disulfide bonds in the molecule (13, 14). Three structurally and kinetically distinct populations of folding intermediates can be purified from JAR choriocarcinoma cells and they have

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1 The abbreviations used are: hCG, human chorionic gonadotropin; HPLC, high-performance liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; CPM, counts per minute; CM-, carboxymethyl-; PE-, pyridylethyl-. Peptide nomenclature: [\([135S\text{Cys}]\)](9–20)-peptide is referred to as \(\beta\)(9–20).
the following precursor-product relationship: pβ1 → pβ2-free → pβ2-combined (12, 15). The differences in the disulfide bonds that each intermediate contains supported an intracellular folding model in which the core disulfide bonds (between cysteines 34–88 and 38–57) form first, followed by bonds that may stabilize proposed β sheet structures (disulfide bonds 9–90 and 23–72), and finally by the formation of disulfide bonds that stabilize the carboxyl terminus of the molecule (between cysteines 93–100 and 26–110) (14).

In a previous paper (14), 6–64% of each of the six disulfide bonds were reported to be unform in pβ1, indicating that this hCG-β population contained a mixture of intermediates. Multiple intermediates within the pβ2-free and pβ2-combined pools were also predicted since partial disulfide bond formation involving cysteines 93 and 100 as well as cysteines 26 and 110 was observed in these hCG-β forms. If folding proceeds by a simple sequential model in the cell, then tertiary intramolecular interactions, including disulfide bonds, would be expected to occur in a discrete order. Two explanations can account for the mixture of hCG-β molecules in the pβ1, pβ2-free, and pβ2-combined populations: (1) disulfide bond stabilization of tertiary structure may not be entirely sequential so that bonds occur independently in different regions, or domains, of the molecule; (2) the multiple intermediates in the pβ1, pβ2-free, and pβ2-combined pools do constitute a sequential folding pathway, but, due to the sampling times previously examined, they were not kinetically separable. Experiments described in this report were designed to distinguish between these possibilities by quantitating disulfide bond formation in kinetic intermediates within the pβ1, pβ2-free, and pβ2-combined pools by means of rapid sampling of [35S]cysteine pulse-labeled cells. Furthermore, by analyzing the kinetics of S-S bond formation of early pβ1 intermediates, we hoped to measure the order in which the earliest disulfide bonds form and to determine whether these bonds form co- or posttranslationally.

EXPERIMENTAL PROCEDURES

Cell Culture—JAR human choriocarcinoma cells were grown at 37 °C in Dulbecco’s modified Eagle’s medium (GIBCO) with 10% fetal bovine serum (Sigma) as described previously (16).

Biological Labeling—Isolation of hCG-β folding intermediates that reflect the folded state of the molecule at defined time points after synthesis was accomplished by pulsing JAR cells with [35S]cysteine and chasing them in medium that contained radioactive cysteine. All cells were starved of cysteine in cysteine-free and serum-free medium for 30 min prior to metabolic labeling. For each time point, four 100-mm Petri dishes of confluent JAR cells (passage 525–550) were labeled for 40 or 120 s with 400 μCi/ml [35S]cysteine in 5 ml of Dulbecco’s modified Eagle’s medium (GIBCO), which lacked cysteine. Cells labeled for 40 s were immediately harvested. Cells labeled for 120 s were chased with nonradioactive medium for 0, 2, 4, 8, or 25 min. 2 μCi of [35S]cysteine (1100 Ci/mmol; Du Pont-New England) were used to label each set of four Petri dishes such that the total incorporation of [35S]cysteine into hCG-β folding intermediates was comparable for each time point that included a 120-s labeling period. To minimize the amount of [35S]cysteine incorporation into hCG-β folding intermediates during the chase, cells were rinsed with PBS at 37 °C between the labeling and chase periods. In one of the three experiments that included the 2, 4, 8, and 25-min chase times, cells were chased in the presence of 100 μg/ml cycloheximide. This concentration of drug has been found to inhibit 90% of protein synthesis. Furthermore, the drug has no effect on the kinetics of folding of the molecule (data not shown). Cells were harvested by lysing them in PBS (pH 8.4–8.7) containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 20 mM EDTA, 2 mM phenylmethanesulfonfluoride, and 50 mM iodoacetate. Cell lysates were rotated for 30 min in the dark at room temperature, and the pH was then reduced to 6.5 with 1 N HCl. The cell lysates were stored at 4 °C until the removal of microsomal membranes by centrifugation (100,000 × g, 60 min).

Purification of Folding Intermediates—This procedure has been described in detail elsewhere (14). Briefly, the pβ2-free population was specifically immunoprecipitated from the JAR cell lysates using a monoclonal antibody (Chemicon). pβ1 and the intracellular dimer that contains the pα and pβ2-combined subunits were next immunoprecipitated with a polyclonal anti-hCG-β antiserum termed HHC. Immune complexes were pelleted from the cell lysates with protein A-Sepharose (Sigma). The beads were washed extensively with cell lysis buffer (pH 6.5) that lacked iodoacetate and then with PBS, pH 6.5. To break the hCG-α-β, antibody-antigen, and antibody-Protein A complexes, the pellets were resuspended in 2 ml of HCl, pH 3 (Pierce, sequencing grade), for 16 h at room temperature with 100 μg of myoglobin (Sigma). Myoglobin was used as a carrier protein throughout these experiments to prevent the loss of hCG-β by sticking to plastic surfaces. hCG-β folding intermediates were purified from the guanidine eluates by reversed-phase HPLC using a Vydac 300-A, 5-μm, C18 reversed-phase column (0.46 × 25 cm). A 90-min, linear 18–50% acetonitrile gradient in 0.1% trifluoroacetic acid (1 ml/min) was used to resolve the folding intermediates (14, 17). Elution positions were confirmed by analyzing 5% aliquots of each 1-min fraction by liquid scintillation counting. Fractions that contained each folding intermediate were concentrated by Speed-Vac centrifugation and pooled.

Nonreducing SDS-PAGE—2.5 or 5% of the protein A-Sepharose-antibody antigen complexes from each immunopurification as well as 5% aliquots of each HPLC-purified folding intermediate wereanalyzedon nonreducing SDS-PAGE as described (14). The radioactive folding intermediates were visualized in the gels in the cold in 1% sodium dodecyl sulfate (New England Nuclear) and exposing the dried gels to x-ray film.

Tryptic Digestions—Nonreduced hCG-β intermediates were digested for 16 h in silanized polypropylene tubes containing 100–200 μg of myoglobin, 50 μg of diphenylcarbamylchloride-treated trypsin (Sigma, T-1095), 5 mM CaCl2, and 50–150 μM Tris-HCl, pH 8.2. The digestion was continued for 4 more hours by adding more of trypsin (67 μg/ml final concentration) in two more aliquots. Disulfide bond rearrangements have been reported to occur during proteolytic digestions at alkaline pH (18, 19). However, because our analysis of the folding pathway was based on nondisulfide-linked cysteines that are not cleaved by trypsin in the cell, the occurrence of disulfide bond rearrangements that might have occurred during the tryptic digestions (14), which were performed in the absence of iodoacetate, do not affect our conclusions. Also, the same trypsin peptide pattern was observed for digests of pβ1 that were performed at pH 6.5 or 8.0 (14), further indicating that disulfide bond rearrangements were unlikely to have affected our results.

HPLC Purification of Tryptic Peptides—Tryptic digests were injected onto a Brownlee C8 RP-300, 10-μm reversed-phase column (0.46 × 25 cm) equilibrated with 0.1% trifluoroacetic acid (buffer A). The column was eluted isocratically for 3 min with buffer A followed by an isocratic gradient for 100 min with buffer B (95% buffer B: 5% buffer A) at 1.0 ml/min. One-min fractions were collected in silanized polypropylene tubes. Tubes into which disulfide-linked peptides eluted contained 45 μg of myoglobin. Samples were concentrated by Speed-Vac centrifugation and stored at −20 or −70 °C.

Ion Exchange HPLC—Two peptides co-eluted on reversed-phase HPLC, β(86–74) and β(105–114), and were subsequently resolved by ion exchange chromatography using a Waters DEAE-anion exchange column (1000-Å pore size, 10 × 100 mm). The peptides were eluted during a 30-min 0–300 mM NaCl gradient at a constant pH of 7 in 20 mM phosphate buffer. One-min fractions containing 1 μl were contaminated by a single disulfide bond rearrangement that might have occurred during the tryptic digestions (14), which were performed in the absence of iodoacetate, do not affect our conclusions. Also, the same trypsin peptide pattern was observed for digests of pβ1 that were performed at pH 6.5 or 8.0 (14), further indicating that disulfide bond rearrangements were unlikely to have affected our results.

HPLC Peptides—HPLC peaks that contained disulfide-linked peptides, which have been previously identified (14), were concentrated by Speed-Vac centrifugation in silanized polypropylene tubes. Reductions were performed in 1.5 ml containing 150 mM Tris-HCl, pH 8.4, and 20 mM dithiothreitol under nitrogen at 37 °C for 3–5 h. Thiols were pyridylethylated with 50 mM vinylpyridine (Aldrich). 8.3 μl of vinylpyridine was mixed with 20 μl of acetonieter before addition to the 1.5-ml reaction mixture. Following addition to the reduction mixture, a single disulfide bond was not formed in the aqueous environment. Tubes were flooded with nitrogen and rotated at room temperature for 16 h. The initial tryptic digestion of the nonreduced folding intermediates did not cleave all of the lysine and arginine peptide bonds, particularly those of the pβ2-free and pβ2-combined subunits (14). Therefore, the reduction and pyridylethylated peptides of each intermediate were digested with trypsin for 2 h at 37 °C. The pyridylethyl-cysteine-containing peptides were
then separated by reversed-phase HPLC on a C18 column and detected by scintillation counting of the [35S]cysteine in each peptide as described above.

Gas Phase Sequencing and Amino Acid Analysis—The peptide β-(83-94) of pβ1 was sequenced according to methods published previously (14). Amino acid analysis (14) of nondisulfide-linked tryptic peptides was used to prove that the latter two bonds formed at a slower rate than 34-88, the release of the peptide β-(87-94) reflects the kinetics of formation of bond 34-88 (Fig. 4).

RESULTS

Identification of [35S]Cysteine-labeled hCG-β Folding Intermediates by SDS-PAGE—The [35S]cysteine-labeled folding intermediates of hCG-β were immunopurified from JAR choriocarcinoma cells that had been labeled with [35S]cysteine for either 40 or 120 s, chased for 0-25 min, and lysed in the presence of iodoacetate. These intermediates were then resolved by nonreducing SDS-PAGE (Fig. 1A). Two antibodies were employed to isolate hCG-β intermediates from detergent solubilized cell lysates: a monoclonal antibody (referred to as α in Fig. 1A) that recognizes only pβ2-free and a polyclonal antibody (referred to as αβ in Fig. 1A) that recognizes pβ1 as well as the αβ hCG dimer. Following 40 or 120 s of metabolic labeling with [35S]cysteine, a slightly slower migrating species of pβ1, termed pβ1-early (Fig. 1A, lanes 2 and 4), was detected that rapidly chased to the M1, 23,000 locus of pβ1, termed pβ1-late (Fig. 1A, lanes 6, 8, and 10). The reason why hCG-β kinetic intermediates migrate differently in nonreducing SDS gels is not clear. Presumably, the different conformations of these intermediates affect the extent of SDS binding that in turn affects the rate of migration in these gels.

The conversions of pβ1-late (Fig. 1A, lanes 6, 8, and 10) to pβ2-free (Fig. 1A, lanes 5, 7, 9, and 12) and of pβ2-free to pβ2-combined (Fig. 1A, lanes 8, 10, and 13) are also shown and reflect the precursor-product relationships previously reported (12, 15). These kinetic studies, the differences in migration on SDS-PAGE, and the changes in antibody reactivity all demonstrate that the structures of these intermediates differ. Thus, the differences in disulfide bonding between the hCG-β intermediates (13, 14) seem to reflect conformational changes during the folding pathway.

Only 2.5 or 5% of the immunopurified hCG-β intermediates was applied to the gels that are shown in Fig. 1A. The remaining material was purified by reversed-phase HPLC as described under “Experimental Procedures.” Purity was confirmed by analyzing the isolated hCG-β intermediates by reversed-phase HPLC. The chymotryptic-like clips at the peptide bonds of Tyrβ2 and Leuβ2 have been described (14). The peptide β-(69-74) and β-(83-94) have been used to prove that the latter two bonds formed at a slower rate than 34-88, the release of the peptide β-(87-94) reflects the kinetics of formation of bond 34-88 (Fig. 4).

### TABLE I

| Disulfide pairing of hCG-β tryptic peptides and elution positions of the peptides on reversed-phase HPLC |
|---------------------------------------------------------------|
| S-S bond          | Disulfide-linked peptides | Peptide released when bond not formed | Elution position, peak number of fig. 2 |
|-------------------|---------------------------|--------------------------------------|----------------------------------------|
| Cysβ2-Cysβ4       | β-(83-94) and β-(21-43)   | β-(83-94) and β-(21-43)               | 5                                      |
| Cysβ2-Cysβ8       | β-(87-94)                 |                                       | 3                                      |
| Cysβ2-Cysβ8      | β-(44-60) and β-(21-43)   | β-(44-60)                            | 2 of Fig. 3                            |
| Cysβ6-Cysβ8      | β-(69-74) and β-(21-43)   | β-(69-74)                            | 2                                      |
| Cysβ6-Cysβ8      | β-(96-104) and β-(83-94)  | β-(96-104)                           | 4                                      |
| Cysβ8-Cysβ8      | β-(105-114) and β-(21-43) | β-(105-114)                          | 2                                      |

* The peptide β-(83-94) is released when three S-S bonds are unformed: 34-88, 9-90, and 93-100. Because the latter two bonds form at a slower rate than 34-88, the release of the peptide β-(87-94) reflects the kinetics of formation of bond 34-88 (Fig. 4).

** The chymotryptic-like clips at the peptide bonds of Tyrβ2 and Leuβ2 have been described (14).

¢ Peptides β-(69-74) and β-(105-114) co-elute on reversed-phase HPLC and are separated by anion-exchange HPLC as described under “Experimental Procedures” and Ref. 14. The ion-exchange chromatograms are not shown in Fig. 2.

¢ Some of this peptide co-elutes with the Tris buffer in peak 1a, the void volume of the column (14).
Domain-dependent Folding of hCG-β

The nonreducing SDS-PAGE analysis of the kinetics of conversion between hCG-β folding intermediates. A, 2.5-5% aliquots of the protein A-antibody-substrate immunocomplexes that were purified from JAR cells labeled for 40 s with [35S]cysteine were chased in the presence of excess nonlabeled cysteine to reveal the kinetics of conversion between the hCG-β intermediates (lanes 4-13) that are identified on either side of the gel. The unassembled form of pP1-free (lanes 2-4) was used to confirm the purity of these forms. B, SDS-PAGE analysis of aliquots of the HPLC-purified pP1 intermediates (lanes 2-4) used to confirm the purity of these forms. C, confirmation of purity of the HPLC-purified pP2-free (lanes 2-5) and pP2-combined (lanes 6-8) intermediates by SDS-PAGE. Lanes 1, 3, 11, and 14 of A, lanes 1 and 5 of B, and lanes 1 and 9 of C contain 14C-labeled standards (Sigma): bovine serum albumin monomer (M, 66,000), chicken egg albumin (M, 45,000), carbonic anhydrase (M, 29,000), α-lactalbumin (M, 14,000).

nonreducing SDS-PAGE (Fig. 1B). Lanes 2 and 3 (Fig. 1B) demonstrate the pP1-early to pP1-late conversion. That pP2-combined was efficiently purified from pP1 and pP0, which were also immunoprecipitated with the G10 (anti-αβ) antibody, was demonstrated by SDS-PAGE (Fig. 1C, note the absence of pP1 and hCG-α bands in lanes 6-8). After a 25-min chase period, mature β was observed (Fig. 1A, lane 13). For the disulfide bond studies of pP2-combined from the 25-min chase time, this intermediate was separated from mature β by HPLC as demonstrated by SDS-PAGE of the purified pP2-combined (Fig. 1C, lane 8).

Strategy to Measure the Folding Pathway(s) of the hCG-β Intermediates—We have previously reported a strategy to identify the order of disulfide bond formation in hCG-β intermediates isolated from JAR cells that had been labeled for 4 min or longer (13, 14). The results showed that detectable conformational changes occur during the intracellular folding pathway of hCG-β. In the present study, we isolated additional folding intermediates from JAR cells labeled for 40 s or 2 min and chased for up to 25 min after metabolic labeling (Fig. 1, B and C). To measure the unformed disulfide bonds, the nonreduced hCG-β folding intermediates were digested with trypsin to allow the separation of nonsulfur-linked peptides from disulfide-linked peptides. Tryptic peptides were purified by reversed-phase HPLC and identified by retention time (14, 17). In this way, the unpaired cysteines that were present in each folding intermediate when the cells were lysed could be identified. The iodoacetate present in the lysis buffer carboxymethylated cysteine thios and prevented disulfide bond formation involving unpaired cysteines in the cell lysate. Table I lists the nondisulfide-linked peptides that can be purified when a particular disulfide bond is not formed.

In this strategy, if a cysteine was not carboxymethylated in the cell lysate, it was concluded that the cysteine was involved in a native intramolecular disulfide bond of the hCG-β subunit in the intact cell. Because only one-half of an unformed disulfide bond is measured using this strategy, two assumptions must be made: first, that the disulfide bond assignments (20) of the mature hCG-β molecule are correct, and second, that no disulfide bond rearrangements occur during folding of the hCG-β subunit.

In regard to the first assumption, three of the six disulfide bonds (26-110, 93-100, and 23-72) of hCG-β are generally accepted (21). However, the other three disulfide bonds (34-88, 38-57, and 9-90) have been difficult to assign based on the technique of Mise and Bahl (20). Data from our laboratory supports the 9-90 disulfide bond assignment since the same amount (30%) of both cysteines 9 and 90 were found to be carboxymethylated in pP1 after a 5-min chase (14). Moreover, similar amounts of Cys34 (18%) and Cys88 (6-18%) were carboxymethylated in pP1. Finally, the S-S-linked 38-57 peptide has hCG receptor binding activity (22), and NMR studies of a homologous synthetic peptide containing alanines instead of cysteines suggest that this peptide forms a loop configuration that would bring cysteines 38 and 57 into approximation (23). Thus, it seems likely that a similar conformation exists in this region of the hCG-β subunit, allowing a 38-57 disulfide bond to form. Taken together, these data strongly suggest that the disulfide bond assignments originally proposed by Mise and Bahl (20) are correct.

The second assumption is that disulfide bond rearrangement does not occur during the folding pathway. At the resolution of these intracellular folding studies (i.e., pulse-chase time points that differ by 2 min), we have not detected significant evidence of disulfide bond rearrangement during the folding pathway of hCG-β. In support of this conclusion, cysteines 9, 57, 88, 100, and 110 were found to become continuously more disulfide bond as folding progressed. If disulfide bond rearrangement occurs in the hCG-β folding pathway, kinetic studies would have been expected to show a transient decrease in the amount that any one of these cysteines is S-S-linked. Such a decrease was not observed (see results of Fig. 4).

Rate of Formation of the Core Disulfide Bonds 34-88 and 38-57—These two disulfide bonds form first in hCG-β as deduced from the disulfide bond analyses of pP1 purified from cells that were labeled with [35S]cysteine for 10 min and chased for 5 min (14). To identify which of these bonds forms first, and to determine whether these bonds form cotranslationally, pP1 was purified from cells that were labeled for 40 s or 2 min (Fig. 1). Fig. 2 shows the results of the purification of the nondisulfide-linked peptides from pP1 that was isolated following a 40-s label with [35S]cysteine (Fig. 2A), a 2-min label with no chase (Fig. 2B), and a 2-min label followed by a 2-min chase (Fig. 2C). Our data indicate that hCG-β was completely synthesized after these short pulse times, because the peptide that contains the most carboxyl-terminal cysteine (Cys119) was shown to be [35S]cysteine-labeled (Fig. 2A, peak 2). Furthermore, the migration of pP1 on SDS-PAGE did not change as the pulse time was increased from 40 to 120 s (Fig. 2A, lanes 2 and 4), which would have been expected if nascent...
chain elongation had been continuing. Because hCG-β seems to have been completely synthesized after 40 s of metabolic labeling, the changes in disulfide bond structure that were measured in these experiments were concluded to have occurred posttranslationally. The small amount of disulfide-linked peptides in hCG-β labeled for 40 s (Fig. 2A) supports the conclusion that few of the cysteines in hCG-β had formed disulfide bridges cotranslationally. As the duration of label and the chase increased, the amount of disulfide bond formation rapidly increased as shown by the increased distribution of [35S]cysteine containing material into the disulfide-linked peptides (Fig. 2, B and C).

The identification of the nondisulfide-linked peptide β-(83-94) (Fig. 2A, peak 5) shows that the disulfide bond between cysteines 34 and 88 was incompletely formed in pP1 isolated from JAR cells that were labeled for 40 s (Table I). The decrease in the amount of this peptide (peak 5, Fig. 2, A–C) that was released relative to the amount of “S-S-linked peptides” indicates a rapid rate of formation of the 34-88 disulfide bond within the first few min of protein synthesis. Although Cys88, Cys58, and Cys59 are found in the peptide β-(83-94), the changes in the amount of release of this peptide following trypsin digestion of pP1 reflect the kinetics of formation of the 34-88 disulfide bond since the other disulfide bonds, 9-90 and 93-100, have been found to form at a slower rate (11, 14). To show that the release of the peptide β-(83-94) did not result from a disulfide bond rearrangement involving the 34-88 disulfide bond, the peptide β-(83-94) (Fig. 2B, peak 5) was sequenced and the amount of CM-Cys88 was determined as described under “Experimental Procedures.” Greater than 90% of Cys88 was carboxymethylated, indicating that the amount of release of this peptide by trypsin was indicative of the amount of the 34-88 S-S bond that was unformed in the intact pP1 molecule.

In order to determine which of the two earliest disulfide bonds, 34-88 and 38-57, forms first, we compared the amounts of peptides containing Cys88 and Cys58 that were released from trypsin digests of kinetically related pP1 intermediates. Following reduction, pyridylethylated, and HPLC purification of the disulfide-linked peptides, the peptide β-(44-60) that contains CM-Cys58 was separated from the same peptide that contains PE-Cys44 (21). Fig. 3 shows the results of these experiments for early forms of pP1. The changing ratios of peaks 1, which contains PE-Cys44, and 2, which contains CM-Cys58, from digests of pP1 that was isolated 40 (A), 120 (B), or 240 s (C) after protein synthesis suggests that the 38-57 disulfide bond forms posttranslationally. Furthermore, quantitation of the rate of formation of the 34-88 and 38-57 S-S bonds (Fig. 4, A and B) suggests that the 34-88 bond is completed earlier than the 38-57 bond.

**Rate of Formation of Each of the Six Disulfide Bonds in hCG-β**—In addition to identifying the first disulfide bond to form in hCG-β, the rate and sequence of subsequent disulfide bond formation were used to predict which domains of the tertiary structure of hCG-β were stabilized and in what order. This order of stabilization should predict whether the tertiary structure of hCG-β forms via a simple sequential pathway or whether different domains of the molecule fold with independent rates. The major populations of hCG folding intermediates (pP1, pP2-free, and pP2-combined) were purified (Fig. 1), and the disulfide bonds of each intermediate were determined according to the strategy that is detailed in Table I. The extent of S-S bond formation was then quantitatively as described under “Experimental Procedures.” A summary of the results of the three independent experiments is shown in Fig. 4.

As noted above, the earliest disulfide bond seems to have formed rapidly as shown by the increased distribution of [35S]cysteine containing material into the disulfide-linked peptides (Fig. 2, B and C). The extent of S-S bond formation was then quantitated as described under “Experimental Procedures.” A summary of the results of the three independent experiments is shown in Fig. 4.

**Fig. 3.** Separation of the peptides PE-β-(44-60) and CM-β-(44-60) from early pP1 intermediates. The disulfide-linked peptides from the tryptic digests of pP1 (shown in Fig. 2) were reduced and pyridylethylated as described under “Experimental Procedures.” HPLC analysis of the reduced peptides, which included CM-β-(44-60) that had co-eluted with the disulfide-linked peptides in the HPLC purifications of Fig. 2, was used to quantitate the relative amounts of this peptide that were either carboxymethylated or pyridylethylated. Confirmation of the alkylation derivative of Cys58 in each peak was confirmed by amino acid analysis (data not shown). The relative amount of [35S]cysteine CPM in peak 2 corresponded to the amount of Cys58 that was not disulfide-linked in the intact pP1 intermediate. A, analysis of the peptide β-(44-60) of pP1 from a 40-s label with [35S]cysteine; B, 2-min label, 0 chase; C, 2-min label, 2-min chase.
formed between cysteines 34 and 88. After a 40-s label, approximately 60% of this bond was formed (Fig. 4A). A 2-min pulse, only 30% of this S-S bond was formed, and by 2–4 min after translation (see the 2-min pulse, 2-min chase time point) this bond was nearly completely intact, indicating that the 34-88 bond formed with a time constant of about 1–2 min. Cysteines 38 and 57 become disulfide-linked at a slightly slower rate. After a 2-min label, 60% of this bond was still unformed (Fig. 4B) compared to the 30% of the 34-88 bond that was unformed at the same time point. The very fast formation of the 38-57 disulfide bond was 2–3 min.

Our previous studies (11, 14) showed that four disulfide bonds are completely formed by the time pp1 is converted to pp2-free: 34-88, 38-57, 9-90, and 23-72. These results suggested that a domain consisting of residues 1–90 undergoes a conformational change that is stabilized by these four disulfide bonds. The results presented here (Fig. 4C) confirm our previous results that the 9-90 S-S bond of pp1 forms at a slower rate than that of the formation of the bonds 34-88 and 38-57, and that the 9-90 bond is completely formed in pp2-free. Similarly, results were observed for the formation of the 23-72 disulfide bond, i.e. a small amount of it may have formed prior to the formation of pp2-free, but this S-S bond was completely formed in pp2-free. The kinetic profiles (Fig. 4, A–C) show that the 9-90 and 23-72 bonds began to form before the bonds 34-88 and 38-57 were completely intact. The overlapping rates of disulfide bond formation may be indicative of nonsequential stabilization of these disulfide bonds or may be due to the presence of both slow and fast folding species of pp1 that are distinguished by the rates of proline isomerization. Another possibility is that hCG-β subunits synthesized during the chase period could have resulted in exaggerated levels of unformed bonds 34–88 and 38–57 at the later time points. Precautions were taken to limit the amount of [35S]cysteine incorporation during the chase period. The cell monolayer was thoroughly rinsed with PBS following removal of the labeling medium, and in one experiment, cells were chased in the presence of 100 μg/ml cycloheximide. However, because of the short pulse-chase time points used, it is difficult to rule out the possibility of some additional biosynthesis during the chase.

In a sequential folding process, intramolecular interactions would be expected to be completed in discrete steps of the folding pathway. By measuring the kinetics of disulfide bond formation within three physically separable populations of hCG-β folding intermediates, pp1, pp2-free, and pp2-combined, we were able to address whether the formation of disulfide bonds occurs in a strict order. The rates of disulfide bond formation involving cysteines 93 and 100, and 26 and 110 (Fig. 4, E and F), indicated that the carboxyl-terminal domain(s) (residues 93–145) of hCG-β were stabilized at a rate that was independent of the stabilization of residues 1–90. The data in Fig. 4, A–D show that the amino acid residues 1–90 were partially stabilized during the folding of pp1-early to pp1-late. By the time pp1-late folded to pp2-free, this domain of hCG-β was significantly stabilized since four disulfide bonds became completely formed (34–88, 38–57, 9–90, and 23–72). The formation of these four disulfide bonds was also indicative of a significant conformational shift, as indicated by migration on SDS-PAGE (Fig. 1A) and by antibody reactivity (12). The fact that the carboxyl-terminal disulfide bonds 93–100 and 26–110 began to form before and continued to form after the disulfide bonds that are necessary for the pp1-late to pp2-free conversion (Fig. 4) suggests that the carboxyl terminus began to assume tertiary structure before folding of amino acid residues 1–90 was completed. For instance, 50% of the 93–100 bond formed within the pp1 population, another 25% in the conversion of pp1 to pp2-free, and the remaining 25% in the conversion of pp2-free to pp2-combined populations (Fig. 4E). Similarly, 25% of the 26–110 disulfide bond formed with the pp1 population, another 25% of these cysteines formed disulfide bridges in the pp1 to pp2-free folding event, and the remaining 50% of the 26–110 S-S bond formed within the pp2-combined population, i.e. after assembly with the α subunit (Fig. 4F). The slower rate of formation of the 26–110 disulfide bond compared to the 93–100 bond demonstrates that within the carboxyl-terminal region of hCG-β, there was a preferred order of stabilization.

**DISCUSSION**

We have previously reported an intracellular folding model of hCG-β that was based on the changes in disulfide bond structure during the conversions between three intracellular intermediates of hCG-β and extracellular mature β: pp1 → pp2-free → pp2-combined → mature β (14). In that study, the completion of the disulfide bonds 23–72 and 9–90 correlated with the conversion of pp1 to pp2-free; completion of the 93–100 disulfide bond correlated with the pp2-free to pp2-combined conversion, and completion of the 26–110 bond correlated with the pp2-combined to mature β conversion. Those results were based on the structures of the intracellular intermediates that were each isolated from cells labeled for 10 min and chased for 5 min. We have extended the analysis of the hCG-β intracellular folding pathway by analyzing earlier intermediates of hCG-β (after a 40- or 120-s pulse) and by analyzing kinetic intermediates within the pp1, pp2-free, and...
pp2-combined populations. The results of the kinetic studies have allowed us to address the question of whether the stabilization of the tertiary structure of hCG-β in a cell occurs via a simple sequential model of protein folding.

In this study, two additional transitions in the intracellular folding pathway of hCG-β were detected, i.e. steps 1 and 4 in the pathway: pβ1-early → pβ1-late → pβ2-free → pβ2-combined early → pβ2-combined late. The first three of these folding events are detectable by SDS-PAGE (Fig. 1). The results presented in Figs. 2 and 3 indicate that within seconds of metabolic labeling (i.e. prior to the first folding step), pβ1-early lacks 60–80% of the disulfide bond 34–88 and nearly all of the other disulfide bonds. Thus, it seems that almost all of the stabilization of tertiary structure by the disulfide bonds of hCG-β occurs posttranslationally. In step one of the folding pathway, which occurs within the first few minutes after translation, the disulfide bond 34–88 forms and is followed by formation of the 35–57 S-S bond. These two disulfide bridges seem to convert pβ1-early to a faster migrating species on SDS-PAGE that we term pβ1-late (Fig. 1B). In step two, the conversion of pβ1-late to pβ2-free, which is the rate-limiting event in the folding pathway (12), the bonds 23–72 and 9–90 completely form (Fig. 4 and Ref. 14). In step three, pβ2-free to pβ2-combined, the 93–100 S-S bond seems to form almost entirely (Fig. 4 and Ref. 14), in agreement with the results of mutagenesis studies (24, 25) that show the necessity of this molecule.

The purpose of the kinetic studies was to see if conformational changes that are indicated by the disulfide bond formation in hCG-β follow a sequential model of protein folding. The conformational changes during the folding of hCG-β that are indicated by the kinetic studies reported here support a model in which two regions of the hCG-β molecule fold independently. Without a three-dimensional structure of this molecule, it is difficult to define the amino acids in each apparent folding domain. However, one domain seems to include residues 1–90, since four disulfide bonds stabilize the structure of this sequence and since a dramatic conformational shift is observed when these four disulfide bonds are completely formed during the transition of pβ1-late to pβ2-free (Fig. 1A and Fig. 4). Within this domain, the order of disulfide bond formation seems to be 34–88, 35–57, and 9–90 together with 23–72. The second domain of hCG-β that folds with an independent rate includes residues 95–145 and is stabilized by the disulfide bonds 93–100 followed by 26–110. Thus, even though the folding pathway of hCG-β cannot be explained by a simple sequential model, there is a definite order of disulfide bond formation within each putative domain of this molecule.

The results presented here are some of the first pieces of evidence to indicate that, in a cell, folding can occur independently in multiple domains of a protein. In another report, the pyridoxal 5′-phosphate binding domain of aspartate aminotransferase, which was produced by recombinant DNA technology from the wild type gene that codes for a two domain protein, was reported to fold to an active state in vivo (28). This indicated that the pyridoxal 5′-phosphate binding domain of the wild type protein could fold independently in a cell. However, most of the evidence for domain-dependent protein folding comes from in vitro refolding studies of proteins such as staphylococcal nuclease (29), lysozyme (30), and cytochrome c (31). Further evidence of a domain mechanism of protein folding comes from theoretical simulations of the folding pathway of the tryptophan synthase α subunit (32) and from analyses of protein x-ray structures to show how submolecular domains can be arranged in a hierarchical folding pathway to generate the native state (7). It is possible that this domain mechanism of protein folding can be found to apply to intracellular folding pathways other than the one described in this report. In support of this prediction, rapid pulse-chase studies to detect posttranslational modifications of viral glycoproteins have revealed the presence of cysteine thiols that become oxidized while in the endoplasmic reticulum (33, 34). Perhaps the intracellular rate and order of disulfide bond formation that occurs for proteins such as these would support a similar domain-dependent protein folding pathway.

Disulfide bond rearrangement has been a hallmark of the bovine pancreatic trypsin inhibitor folding pathway (4–6). Although our analyses of the hCG-β folding intermediates are not as complete as the analyses of bovine pancreatic trypsin inhibitor folding intermediates, we have not found disulfide bond rearrangement to be an integral part of the hCG-β form. 4) pβ1 is more easily digested with trypsin than pβ2-free and pβ2-combined, indicating a less compact form when few S-S bonds are present. 5) There is a step-wise progression of S-S bond formation that parallels the conversion of an assembly-incompetent to an assembly-competent conformation of the hCG-β subunit (12). Taken together, these data indicate that the disulfide bridging of hCG-β in a cell is coincident with the folding process and reflects the conformational state of the molecule.
folding pathway. Once the cysteines of hCG-β have formed disulfide bridges, they do not seem to rearrange during the conversions between folding intermediates. If rearrangement of S-S bonds involving cysteines 88, 100, 72, 110, or 9 does occur at the time scale of these experiments (i.e., min), then the percent that a cysteine is unformed would be expected to oscillate in the kinetic experiments (Fig. 4). This is not the case. All of the disulfide bonds of hCG-β form in a unidirectional manner (Fig. 4).

Trapping the free thiols of cysteine by alkylation is an effective method to preserve the disulfide bond structure of a folding intermediate. However, disulfide bond rearrangements can occur at a similar rate as the alkylation reaction (4, 5), and incorrect conclusions concerning the folding pathway can be made if such disulfide rearrangements result in bonds that do not really occur in the protein during folding. We previously reported that the same unformed disulfide bonds were found in the folding intermediates whether cells were lysed at pH 6.5, where disulfide bonds are less likely to rearrange (17), or at pH 8.0, both in the presence of iodoacetate (14). Based on those results, we do not believe that disulfide bond rearrangements occurred prior to thiol alkylation of hCG-β in the detergent solubilized cell lysates.

In summary, our studies show that the formation of disulfide bonds reflects conformational changes that occur in hCG-β during folding in intact cells. We have found that the formation of disulfide bonds of hCG-β in cultured cells is unidirectional with time and occurs posttranslationally. However, because folding seems to occur independently in two domains of the molecule, the folding pathway of hCG-β cannot be explained by a simple sequential model. Finally, no significant amount of nonnative disulfide bonds have been found to form during folding of hCG-β in JAR choriocarcinoma cells.

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