Molecular chaperones facilitate the folding of proteins in the endoplasmic reticulum (ER) of mammalian cells. The glycoprotein hormone chorionic gonadotropin β subunit is a secretory protein whose folding in the ER has been demonstrated (Huth, J. R., Mountjoy, K., Perini, F., and Ruddon, R. W. (1992) J. Biol. Chem. 267, 8870–8879). Because folding of wild type hCG-β subunit occurs in the ER with a $t_{1/2} = 4–5$ min, stable association of ER chaperones with hCG-β have been difficult to detect probably because they have a short half-life. However, β-chaperone complexes containing the ER chaperones BiP, ERP72, and ERP94 have been detected in slow folding mutants of hCG-β subunit that lack both of the N-linked oligosaccharides (Feng, W., Matzuk, M. M., Mountjoy, K., Bedows, E., Ruddon, R. W., and Boime, I. (1995) J. Biol. Chem. 270, 11851–11859). The questions addressed here are 1) whether the detection of chaperone complexes is related to the absence of carbohydrate or to the rate of hCG-β subunit folding, 2) whether such complexes are dead-end or whether they lead to formation of a secreted, mature hCG-β form, and 3) what the nature of the hCG-β-chaperone binding is. The data obtained indicate that the amount of detectable hCG-β-chaperone complexes correlates with the rate or extent of folding, that the complexes of hCG-β with ER chaperones lead to the formation of secretable β, and that the complexes of hCG-β with chaperones involve the formation of intermolecular disulfide bonds.

In the endoplasmic reticulum (ER) two classes of proteins assist polypeptide folding. These proteins include folds such as peptidylprolyl cis-trans-isomerase and protein disulfide isomerase, which catalyze rate-limiting isomerization steps in protein folding, and binding proteins called molecular chaperones that stabilize unfolded or partially folded structures and prevent the formation of inappropriate folding interactions (1–3).

Molecular chaperones are proteins that are expressed in cellular compartments where protein folding occurs, and those of the heat-shock protein families (e.g. hsp-70 and hsp-90) are frequently induced to higher levels during response to stress. They function in protein folding and translocation by mechanisms that involve binding to hydrophobic surfaces of unfolded proteins. This prevents aggregation and fosters conformational changes that lead to native structure (1–3). Chaperone-mediated protein folding appears to proceed by multiple rounds of binding and release of nonnative forms (4) and by sequential binding of various chaperones that may be involved in different steps of the folding pathway (5). However, binding of partially folded intermediates to chaperones can facilitate protein folding while the folding intermediate is still chaperone-bound (6).

The molecular chaperones of the hsp-70 and hsp-90 families in eukaryotic cells function in the stabilization, translocation, and degradation of partially folded intermediates during polypeptide folding and assembly. BiP is a member of the hsp-70 chaperone family in the ER of eukaryotic cells. It has been proposed that BiP associates transiently with unfolded or misfolded proteins to modulate protein folding (5, 7). ERP94, a member of hsp-90 chaperones, appears to function with BiP to assist protein folding in the ER lumen (8). ERP72 has been identified as an ER protein containing protein disulfide isomerase homology units (9). In addition, calnexin (also called p88, IP90), an ER transmembrane protein, plays a chaperone-like role by binding with monoglucose-containing N-linked glycans of newly synthesized glycoproteins (5, 10). Calreticulin, another ER Ca$^{2+}$-binding protein, has also been implicated as a molecular chaperone (9, 11).

The folding of the β subunit of hCG has proven to be an excellent model for studying the events that lead to the production of a biologically active hormone (12, 13). It has been reported to be the only mammalian protein for which a folding pathway in intact cells has been established (14). Because it is a secretory glycoprotein with six intramolecular disulfide bonds and must assemble with the glycoprotein hormone α subunit to become biologically active, several lessons may be learned about the role of disulfide bonds, oligosaccharides, and chaperones in the in vivo folding, assembly, and secretion of such proteins. Using the formation of disulfide bonds as an index of conformational changes during protein folding, the intracellular kinetic folding pathway of the β subunit of hCG has been determined (12, 13, 15), as shown below with the disulfide bonds formed between Cys residues (e.g. Cys$^{34}$–Cys$^{88}$) at each step indicated above these arrows:

\[
\begin{align*}
34–88 & \rightarrow 38–57 \\
9–90 & \rightarrow 23–72 \\
93–100 & \rightarrow 26–110
\end{align*}
\]

The abbreviations used are: ER, endoplasmic reticulum; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; WT, wild type.
Wild type hCG-β subunits recovered from the ER possess two high mannose-containing asparagine (N-linked) oligosaccharyl side chains at residues Asn13 and Asn30 (16). We have previously examined the kinetics of hCG-β folding in Chinese hamster ovary (CHO) cells transfected with wild type or mutant hCG-β genes lacking either or both N-linked glycosylation sites (17) as well as CHO cells containing hCG-β genes mutated at each of the cysteine involved in the formation of the six intramolecular disulfide bonds (18, 19). Folding of the β subunit is inhibited or slowed with many of these mutants. In the case of the glycosylation mutants, we found that the $t_\text{50}$ for folding of the first detectable β folding intermediate, $p_{11}$, into the second major hCG-β folding intermediate, $p_{22}$ (the rate-determining step in hCG-β folding), was 5–7 min for wild type β but 33 min for β lacking both N-linked glycans. However, the $t_\text{50}$ of conversion of $p_{11} \rightarrow p_{22}$ was 7–8 min in CHO cells expressing hCG-β subunits missing only the Asn13-linked glycan and 10 min for hCG-β subunits missing only the Asn30-linked glycan (17). Moreover, we reported that the ER chaperones BiP, ERP74, and ERP94, but not calnexin, are co-immunoprecipitated with unglycosylated hCG-β folding intermediates (17).

In this report, we examine whether the formation of stable complexes of hCG-β with ER chaperones occurs in several CHO cell lines transfected with the WT β gene or a variety of mutated hCG-β genes: 1) slow folding mutants in which both Cys residues of intramolecular disulfide bonds were replaced by Ala, 2) glycosylation mutants in which Asn residues of N-linked glycosylation sites were converted to Gln, or 3) a previously undescribed CHO cell line transfected with a Pro $\rightarrow$ Gly mutant of hCG-β (P73G) that slows hCG-β folding. We have determined that the amount of hCG-β-chaperone complex correlates with the rate or extent of hCG-β subunit folding. The slower folding mutants formed a greater detectable amount of chaperone complex, but the nonfolding mutants did not form detectable amounts of chaperone complex. Moreover, the hCG-β-chaperone complexes appear to be productive in that hCG-β contained in them is ultimately folded and secreted from CHO cells. Finally, the hCG-β-chaperone complexes involve the formation of intermolecular disulfide bonds.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The terminology used here is: CHO β WT, β Asn1, β Asn2, and β Asn1/2 for CHO cells transfected with wild type hCG-β genes or hCG-β genes containing mutations Asn→Gln, at Asn13, Asn30, or both glycosylation sites, respectively (20). CHO βC9A/C90A, βC38A/C57A, and βC23A/C72A refer to cells in which Cys9 and Cys90, Cys38 and Cys57, and Cys23 and Cys72 residues were replaced by Ala residues (18). These stably transfected CHO cells were grown and maintained in Ham's F-12 medium supplemented with the antibiotic G418 as described previously (17). CHO β P73G denotes the conversion of the hCG-β Pro73 residue to a Gly residue. CHO P73G cells were transfected with the pg5 plasmid (a gift of Dr. Tyler White, Scis Nova, Mountainview, CA) and were grown and maintained in UltraCulture medium (BioWhittaker, Inc) supplemented with 50 μM methionine sulfoximine.

**Bioisotopic Labeling**—CHO cells were metabolically labeled as described previously (17). Briefly, 100-mm Petri dishes of 90% confluent CHO cells were starved in cysteine-free and serum-free Dulbecco's modified Eagle's medium without G418 for 30 min. These cells were then pulse-labeled for 5 min with 200–400 μCi/ml L-[35S]cysteine (1100 Ci/mmol; DuPont NEN) in serum-free Dulbecco's modified Eagle's medium (Life Technologies, Inc) without G418 and lacking cysteine and chased for the times indicated in the figure. The cells were rinsed with cold phosphate-buffered saline and lysed in 5 ml of phosphate-buffered saline (pH 8.0) containing detergents (1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS), protease inhibitors (20 μM EDTA and 2 mM phenylmethylsulfonyl fluoride), and 50 μM sodium iodoacetate (pH 8.3) to alkylate free sulfhydryl groups of folding intermediates and to prevent further disulfide bond formation or rearrangement.

Immunoprecipitation of Cell Lysates and Separation of β Folding Intermediates by SDS-PAGE—The cell lysates or chase media were immunoprecipitated with an anti-hCG-β polyclonal antibody (1:1000), which recognizes all forms of the hCG-β subunit, for 16 h at 4°C, and the immune complexes were precipitated with protein A-Sepharose (Sigma), as described previously (17).

The protein A-Sepharose-hCG-β immune complexes were eluted with 2 x concentrated SDS-PAGE sample buffer (125 mM Tris-HCl, 2% SDS, 20% glycerol, and 4 μg/ml bromphenol blue) and analyzed by SDS-PAGE using reducing SDS-PAGE as described previously (12, 13). Gel images were prepared using a BioImage® 110S Image Analyzer equipped with a Kodak Charged Caption Device camera, Whole Band Software (Millipore), and a Seiko Instruments model CH-5504 color printer as described previously (19).

**Purification of hCG-β Folding Intermediates**—The protein A-Sepharose-bound immune containing hCG-β immune complexes were eluted with 6 M guanidine hydrochloride (pH 3) (Sequana grade; Pierce) for 16 h with rotation at room temperature. Eluates were purified by reversed-phase high performance liquid chromatography (HPLC) using a Vydac 300-Å C4 column with elution by an acetonitrile gradient as described previously (12). The fractions containing hCG-p11 or p22 subunits and hCG-chaperone-β complexes were collected and concentrated by SpeedVac® concentrator as described previously (17).

**SDS-PAGE and Western Blot Analysis**—To detect the presence of proteins that co-immunoprecipitated with β folding intermediates, CHO β WT, β Asn1, β Asn2, or β Asn1/2 cell lysates were immunoprecipitated with polyclonal hCG-β antisera, and the hCG-β subunits were eluted from protein A-Sepharose beads with 6 M guanidine (pH 3) for 16 h with rotation at room temperature and purified by C4 reversed-phase HPLC (12, 17). The 80–95 min and 100–107 min fractions (termed C1 and C2, respectively) containing β-chaperone complexes were collected and separated by SDS-PAGE under reducing conditions. The resolved proteins were transferred to Immobilon polyvinylidene difluoride transfer membranes (Millipore) in a Trans-Blot apparatus (Bio-Rad) at 400 mA for 1–2 h at 4°C. The membranes were immunoblotted with either rat anti-BiP (1:500), rabbit anti-ERP72 (1:100), rabbit anti-ERP94 (1:100), or rabbit anti-calnexin (1:2000) overnight at 4°C with gentle shaking. Rat anti-BiP polyclonal antibody was kindly provided by Dr. David Bole (University of Michigan, Ann Arbor, MI). Rabbit anti-ERP72 (against the 16 C-terminal amino acids of murine ERP72 and rabbit anti-ERP94 (against the 16 C-terminal amino acids of murine ERP94) were provided by Dr. Michael Green (St. Louis University Medical Center, St. Louis, MO). The rabbit anti-calnexin (against the C-terminal 19 amino acids of canine calnexin) was provided by Dr. Ari Hellenius (Yale University, New Haven, CT). Membranes were washed several times with buffer containing 20 mM Tris, 150 mM NaCl, 1% nonfat milk, and 0.2% Tween (pH 7.4) and incubated with anti-rat or anti-rabbit IgG peroxidase conjugate (1:1000, Sigma) (pH 7.4) and incubated with anti-rat or anti-rabbit IgG peroxidase conjugate (1:1000, Sigma) at room temperature. Eluates were purified by reversed-phase high performance liquid chromatography (HPLC) using a Vydac 300-Å C4 column with elution by an acetonitrile gradient as described previously (12). The fractions containing hCG-p11 or p22 subunits and hCG-chaperone-β complexes were collected and concentrated by SpeedVac® concentrator as described previously (17).

**RESULTS**

**Molecular Chaperones Are Involved in Unglycosylated hCG-β Subunit Folding**—To investigate the kinetics of association of molecular chaperones with β folding intermediates, CHO cells, transfected with the wild type (β WT) or a mutated hCG-β gene (β Asn1/2) containing mutations N130 and N300 at the two hCG-β N-linked glycosylation consensus sequences (20), were pulse-labeled for 5 min with [33S]cysteine and chased for periods of 0–120 min. The cell lysates were then immunoprecipitated with polyclonal hCG-β antisera followed by protein A-Sepharose precipitation. Bound immunocomplexes were eluted with 6 M guanidine and purified by C4 reversed-phase HPLC. As previously seen (17), wild type p11 (Fig. 1A) converted to p22 more efficiently than unglycosylated p11 (p110) (Fig. 1, B-D). Two additional peaks, C1 and C2, were more prevalent in the HPLC profile of the β Asn1/2 mutant (Fig. 1, B-D) than...
in that of WT β (Fig. 1A). The new point that we make here is that the C1 and C2 complexes are present as early as 5 min into the chase before much pβ1 is formed and that the C1 and C2 complexes disappear with the same kinetics as folding of pβ1, pβ2, pβ1, and C2, as chaperone-like proteins.

To determine whether the absence of both N-linked oligosaccharides was necessary to form the C1 and C2 hCG-β complexes, a similar experiment to that shown in Fig. 1 was carried out with CHO cells containing the β subunit gene mutated at only one N-linked glycosylation site (either the Asn13 or Asn30 codon). CHO cells containing the wild type β gene or these mutants were pulse-labeled for 5 min with [35S]Cys and chased for 0–120 min. The cell lysates were immunoprecipitated with anti-hCG-β polyclonal antibody for 16 h at 4°C, and the immune complexes were precipitated with protein A-Sepharose. hCG-β immunocomplexes were eluted from protein A-Sepharose beads with 6 M guanidine hydrochloride (pH 3) for 16 h at room temperature. Eluates were analyzed by C4 reversed-phase HPLC (see “Experimental Procedures”). α, β WT pulse-labeled for 5 min and chased for 5 min; B, β Asn(1–2) pulse-labeled for 5 min and chased for 5 min; C, β Asn(1–2) pulse-labeled for 5 min and chased for 30 min; D, β Asn(1–2) pulse-labeled for 5 min and chased for 120 min. pp1, pp1, with both N-linked glycans; pp2, pp2 with both N-linked glycans; pp1low, pp1 lacking both N-linked glycans; pp2low, pp2 lacking both N-linked glycans; C1 and C2, protein complexes containing hCG-β and chaperone-like proteins.

FIG. 1. Isolation of chaperone-hCG-β complexes from CHO β WT and CHO β Asn(1–2) cell lysates. CHO cells transfected with the wild type or mutated hCG-β genes containing Asn → Gln mutations at the Asn13 and Asn30 codons of the two N-linked glycosylation consensus sequences were metabolically labeled with [35S]sulfate for 5 min and chased for 0–120 min. The cells were lysed in 5 ml of phosphate-buffered saline detergent solution (pH 8) containing 50 mM iodoacetic acid (see “Experimental Procedures”). Cell lysates were immunoprecipitated with an anti-hCG-β polyclonal antibody for 16 h at 4°C, and the immune complexes were precipitated with protein A-Sepharose. hCG-β immunocomplexes were eluted from protein A-Sepharose beads with 6 M guanidine hydrochloride (pH 3) for 16 h at room temperature. Eluates were analyzed by C4 reversed-phase HPLC (see “Experimental Procedures”). A, β WT pulse-labeled for 5 min and chased for 5 min; B, β Asn(1–2) pulse-labeled for 5 min and chased for 5 min; C, β Asn(1–2) pulse-labeled for 5 min and chased for 30 min; D, β Asn(1–2) pulse-labeled for 5 min and chased for 120 min. pp1, pp1, with both N-linked glycans; pp2, pp2 with both N-linked glycans; pp1low, pp1 lacking both N-linked glycans; pp2low, pp2 lacking both N-linked glycans; C1 and C2, protein complexes containing hCG-β and chaperone-like proteins.

To quantitate the amount of hCG-β folding intermediates and as Chaperone Complexes in WT β WT and Mutant β-containing CHO Cells—To quantitate the amount of hCG-β forms present as pp1 and pp2 folding intermediates or contained in C1 and C2 hCG-β complexes, experiments similar to those described in Fig. 3 were carried out with additional slow folding or nonfolding hCG-β mutants. CHO cells were pulsed for 5 min with [35S]Cys and carried out with additional slow folding or nonfolding hCG-β mutants. CHO cells were pulsed for 5 min with [35S]Cys and chased for 5, 30, or 120 min. The cell lysates were immunoprecipitated with anti-hCG-β antibody (1:1000) followed by protein A-Sepharose precipitation. The immunocomplexes were eluted from protein A-Sepharose beads with 6 M guanidine and purified on reversed-phase HPLC. The C1 complex from the HPLC (fraction 80–95) was analyzed by reducing SDS-PAGE and transferred to polyvinylidene difluoride membranes for Western blotting. The membranes were cut horizontally by using a prestained molecular weight marker (Bio-Rad) as a guide. The top portion of the membranes containing higher molecular weight proteins (Mr, > 35,000) were probed using antibodies against three ER molecular chaperones: BiP, Mr = 78,000 (panel A, I), ERp72, Mr = 72,000 (panel B, I), and ERP94, Mr = 94,000 (panel C, I). The bottom portion of the membrane was subjected to Western blot analysis using hCG-β antibody (deglycosylated hCG-β Mr, ~ 18,000 (panels A–C, II)). Nonimmune sera were used as controls and demonstrated the absence of bands at the loci detected by the specific chaperone or β antibodies (data not shown).

FIG. 2. Association of BiP, ERP94, and ERP72 with unglycosylated β folding intermediates using Western blot analysis. CHO β Asn(1–2) cells were pulse-labeled with [35S]sulfate for 5 min and chased for 5, 30, or 120 min. Cell lysates were immunoprecipitated with anti-hCG-β antibody (1:1000) followed by protein A-Sepharose precipitation. The immunocomplexes were eluted from protein A-Sepharose beads with 6 M guanidine. The immunocomplexes were precipitated with Protein A-Sepharose. Relative amount of hCG-β was detected using a BiP, ERp72, and ERP94 (Fig. 2). The C2 fraction also contained these chaperones as well as hCG-β and chaperone-like proteins. The amount of hCG-β was detected using BiP, ERp72, and ERP94 (Fig. 2).
Fig. 3. Detection of hCG-β-chaperone complexes. CHO cells (β WT, β Asn1, β Asn2, β Asn(1-2)) were radiolabeled with [35S]cysteine for 5 min and chased for 5 min. The polyclonal anti-hCG-β antibody was used to immunoprecipitate cell lysates. Immunocomplexes were eluted from protein A-Sepharose beads and purified on C18 reversed-phase HPLC. Panel A, β WT; panel B, β Asn1; panel C, β Asn2; panel D, β Asn(1-2). pβ1, pβ1 lacking the Asn1-linked glycan; pβ2, pβ2 lacking the Asn1-linked glycan; pβ2*, pβ2 lacking the Asn2-linked glycan; pβ2**, pβ2 lacking both N-linked glycans; pβ2*, pβ2 lacking both N-linked glycans; C1 and C2, protein complexes containing hCG-β and chaperone-like proteins.

Fig. 4. Quantitation of pβ1, pβ2, C1, and C2 for various hCG-β mutants. CHO cells (β WT, β Asn1, β Asn2, β Asn(1-2), P73G, C23A/C72A, C38A/C57A, and C9A/C90A) were radiolabeled and lysed, and the eluted immunocomplexes were chromatographed as shown in Fig. 3. The resulting HPLC [35S]-labeled radiochromatograms were analyzed by summation of the radioactivity detected in fractions 38-53 (pβ2, open column), 54-77 (pβ1, hatched column), and 80-102 (C1+C2, filled column). The mean value ± standard deviation of the percentage of the total radioactivity of each form depicted in the respective chromatograms is shown. Lane 1, β WT; lane 2, β Asn1; lane 3, β Asn2; lane 4, β Asn(1-2); lane 5, P73G; lane 6, C23A/C72A; lane 7, C38A/C57A; lane 8, C9A/C90A. The number of experiments used to calculate the mean ± S.D. are as follows: n = 3 for WT, β Asn1, β Asn2, and β Asn(1-2); n = 2 for P73G and C23A/C72A; n = 1 for C38A/C57A and C9A/C90A. Only a single experiment is shown for the latter two mutants because no conversion of pβ1 to pβ2 has been observed in several previous experiments (18, 19) and no C1 or C2 peaks were noted in duplicate experiments (not shown). The S.D. bars for the pβ2 values of the β Asn2 (lane 5) and C23A/C72A (lane 6) mutants were too small (± 1.5) to show a deviation from the mean. The P values (by Student’s t test) for the WT and oligosaccharide mutants are as follows: WT versus Asn1, p > 0.2; WT versus Asn2, p > 0.1; WT versus Asn(1-2) p < 0.01; Asn1 versus Asn(1-2), p > 0.05 < 0.10; Asn2 versus Asn(1-2), p < 0.01.

Unglycosylated β Is Released from Chaperones and Secreted—When the 2- or 24-h chase media were collected from [35S]Cys-labeled, β Asn(1-2)-containing CHO cells, immunopurified, and separated by HPLC, only forms that elute at the column void volume were observed (Fig. 6). Neither the C1 and C2 complexes nor degradation products of β were observed in the chase media. Previous studies have shown that most of the C1 and C2 complexes disappear from intracellular lysates by 5 h, the time at which most of the hCG-β forms present in these complexes have been converted into pβ2 and before there is any...
evidence of intracellular degradation of hCG-β (17). Thus, the kinetic precursor-product relationship between the disappearance of the C1 and C2 complexes and the appearance of the pβ2 folding intermediate as well as the inability to detect these complexes in the culture medium even after 24 h indicates that the C1 and C2 complexes were dissociated and suggests that association of hCG-β with chaperones is a transient step in the folding pathway of unglycosylated hCG-β subunits. The two secreted β peaks seen in Fig. 6 represent pβ2-like, folded forms that appear to differ in the amount of alkylation by iodoacetate.  

**DISCUSSION**

The data presented here extend a previous observation that hCG-β subunits lacking both N-linked oligosaccharides form tight complexes with ER chaperones (17) and further demonstrate that WT β and β subunits lacking only one of the two N-linked chains form similar complexes that include the ER chaperone BiP. The amount of the C1 and C2 complexes containing monoglycosylated β and chaperones were intermediate between wild type β and unglycosylated β and appeared to be related to the rate at which these mutant β forms fold in transfected CHO cells. The mutant lacking both N-linked glycans has the slowest t½ of folding (33 min) and the highest amount of C1 and C2 hCG-β-containing complexes (Figs. 3 and 4).

These data raise the question whether the amount of β-chaperone complexes is related to the rate of folding or to the lack of N-linked glycans, which may simply allow for the availability of more hydrophobic sites to bind the chaperones. To test this, other slow folding or nonfolding mutants of β were examined for the amount of β-chaperone complexes formed in β gene-transfected CHO cells (Fig. 4). The data indicate that the cysteine mutants β C38A/C57A and β C9A/C90A, which do not fold to form pβ2, do not form detectable, stable β-chaperone complexes (Fig. 4). Table I summarizes the relationship of the rate of hCG-β folding (pβ1-pβ2), the relative amount of hCG-β contained in the C1 and C2 complexes, and the relative amount of BiP present in the C1 and C2 complexes. These results
indicate that the rate or extent of β folding determines the amount of stable binding of hCG-β to chaperones. Although the chaperone primarily probed for in the complexes formed by the various β forms was BiP (Fig. 5), it is likely that other ER chaperones including ERP72 and ERP94 are also present in the C1 and C2 β-chaperone complexes formed with WT β and the P73G mutant because these complexes separate by HPLC with elution times identical to those containing these chaperones and unglycosylated β (data not shown).

The C1 and C2 β-chaperone complexes are not just dead-end complexes leading to β degradation because these disappear as pβ1 is converted into pβ2 (Fig. 1) at a time when total recovery of β does not change and no β degradation products are detected (17). Furthermore, unglycosylated β does fold efficiently, though more slowly than wild type, into a mature, secretable form (Figs. 1 and 6).

Interestingly, the C1 and C2 unglycosylated β-chaperone complexes survive treatment with 0.1% SDS (during the CHO cell lysis procedure) and 6 M guanidine (during the elution step of immunoonjugates from protein A-Sepharose beads) and boiling in 1% SDS prior to SDS-PAGE (Fig. 5). These results indicate that the C1 and C2 β-chaperone complexes are covalently linked forms. This was demonstrated by the fact that these complexes were not dissociated by reduction (Fig. 5), suggesting that intermolecular disulfide bonds are formed between unfolded β and ER chaperones or that lattices of β oligomers are formed by intermolecular disulfide bonds, thus trapping chaperones in these lattices. There is precedent for the formation of such intermolecular disulfide bond-linked hCG-β oligomers being formed by unfolded or incompletely folded forms of β. For example, we have previously shown that cysteine-mutant forms of hCG-β C34A/C89A, C38A/C57A, and C9A/C90A, which do not fold into pβ2, form such oligomers (19). However, the fact that these mutants do not form detectable C1 and C2 complexes does not support the lattice hypothesis but rather supports the idea that intermolecular disulfide bonds are formed between hCG-β and chaperones.

Wild type hCG-β also forms a BiP-containing complex (Fig. 5A), suggesting that these tight complexes are involved in the folding pathway of WT β as well. It is not clear why the C23A/C72A mutant forms only a small amount of stable C1 and C2 complexes because this mutant also folds, albeit incompletely, to form pβ2. It may be that the cysteines at positions 23 and 72 are involved in the intermolecular disulfide bonds formed between β and the chaperones or that this mutant has a conformation that does not favor stable interaction with chaperones. The mutants that do not fold from pβ1 to pβ2 (C38A/C57A and C9A/C90A) most likely do not form stable C1 and C2 complexes because these mutant β forms do not achieve a conformation that allows stable binding to chaperones, which may relate to the fact that they do not progress down the folding pathway.

When protein folding occurs under suboptimal conditions, for example, when cellular ATP levels are depleted (21) or when BiP or protein disulfide isomerase are present in stoichiometric amounts in relation to substrate (22), disulfide-linked aggregates of substrates can occur. In some instances, these disulfide-cross-linked aggregates can be rescued by restoring more favorable folding conditions, e.g., by adding ATP (21). We are proposing here is unique in that our data suggest that disulfide-linked substrate-chaperone complexes are normal intermediates in the folding of wild type proteins to their native structures.

Finally, the possible differences between the C1 and C2 hCG-β-chaperone complexes should be noted. It is not clear why these two complexes migrate differently on reversed-phase HPLC because they both contain hCG-β and the three chaperones BiP, ERP72, and ERP94 (17). There are some differences, however, between the C1 and C2 complexes. For example, the high molecular weight bands seen by SDS-PAGE are somewhat different between the two fractions (Fig. 5, B–E), suggesting that the array of chaperones or other binding proteins present in the two complexes may differ. Thus, it will be interesting to determine if there is a precursor-product relationship between the hCG-β forms contained in the two complexes and whether the different chaperones contained in the C1 and C2 complexes act at different steps in the folding pathway.

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