REVIEW

Protein folding in the endoplasmic reticulum: Lessons from the human chorionic gonadotropin β subunit

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Abstract

There have been few studies of protein folding in the endoplasmic reticulum of intact mammalian cells. In the one case where the *in vivo* and *in vitro* folding pathways of a mammalian secretory protein have been compared, the folding of the human chorionic gonadotropin β subunit (hCG- β), the order of formation of the detected folding intermediates is the same. The rate and efficiency with which multidomain proteins such as hCG- β fold to native structure in intact cells is higher than *in vitro*, although intracellular rates of folding of the β subunit can be approached *in vitro* in the presence of an optimal redox potential and protein disulfide isomerase. Understanding how proteins fold *in vivo* may provide a new way to diagnose and treat human illnesses that occur due to folding defects.

Keywords: disease states related to protein misfolding; hCG- β subunit as a model of secretory protein folding; molecular chaperones; protein misfolding; role of disulfide bonds and N-linked glycosylation in protein folding, assembly, and secretion

Protein folding of secreted proteins

A growing number of human diseases are now known to be related to protein folding defects, and thus it is important to understand the rules that govern protein folding *in vivo*. The focus of this review is the mechanisms that regulate protein folding in intact cells, particularly those proteins synthesized in the endoplasmic reticulum (ER) of mammalian cells and targeted for translocation to the cell surface or for secretion.

Many of the eukaryotic proteins whose folding and assembly have been studied *in vivo* are membrane or secreted proteins. They follow a similar route to the cell surface. (1) Synthesis occurs in the rough ER. (2) Nascent proteins are translocated into the cisternal space of the ER where the signal peptide is cleaved; initial co-translational folding involving secondary structure and some native tertiary structure occurs; addition of high-mannose N-linked oligosaccharides and initial processing of N-linked

oligosaccharide chains (for glycoproteins) takes place; formation of disulfide bonds occurs, and for multimeric proteins, oligomerization or subunit assembly is attained along with achievement of native structure. (3) The proteins destined for the cell surface or secretion are translocated to the Golgi apparatus, further processed, and then either translocated to the cell surface or packaged into secretory vesicles for secretion.

There are some similarities as well as differences between intracellular protein folding and protein folding in test tubes. For instance, for the tailspike protein of *Salmonella typhimurium* phage P22 (Fuchs et al., 1991; Mitraki et al., 1991) and the human chorionic gonadotropin β subunit (hCG- β) (Huth et al., 1993), the intermediates in the folding pathway for proteins appear to be the same *in vivo* and *in vitro*, but the rate and efficiency with which proteins achieve final native state *in vivo* is higher than *in vitro*. There are also many observations both *in vivo* and *in vitro* showing that correct folding is in competition with misfolding and aggregation. However, in contrast to what happens *in vitro*, cells minimize or circumvent the off-pathway events by helper factors that facilitate the folding process. These co-factors include enzymes such as protein disulfide isomerase

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(PDI) and peptidyl prolyl *cis/trans* isomerase as well as molecular chaperones (e.g., BiP, ERp72, GRp94, and calnexin) that foster folding by keeping proteins on-pathway and by preventing aggregation and other unfavorable interactions.

An obvious advantage of *in vitro* studies is the ability to use physical chemical techniques such as CD, NMR, and electrospray mass spectroscopy to follow protein folding. Studies on a wide variety of proteins have led to the following general scheme of *in vitro* folding events (reviewed in Dill, 1990; Jaenicke, 1991; Ptitsyn, 1991; Lattman & Rose, 1993; Matthews, 1993; Dobson et al., 1994; Richardson, 1994): (1) collapse of the extended (unfolded, reduced, denatured) protein chain into a more compact form containing a hydrophobic core and some secondary structure (e.g., α helices, β strands, β turns, etc.) (<10 ms timeframe); (2) further development of secondary structure and collapse into a more compact "molten globule" (Ptitsyn, 1991) that has partial tertiary structure and retains hydrated nonpolar amino acid side chains as well as a hydrophobic core (5–1,000 ms timeframe); (3) attainment of native tertiary structure involving amino acid side-chain packing and final hydrogen bond rearrangements. For some proteins, formation and rearrangement of disulfide bonds occur during their folding. For monomeric, single-domain proteins, achieving native structure may take seconds to minutes, but, for more complex multi-domain proteins, it may take hours *in vitro*.

To what extent results obtained for *in vitro* folding may be extrapolated to folding *in vivo* is an important question, because the conditions for protein folding inside living cells are very different from those that may be achieved in test tubes. In fact, the above described model of *in vitro* protein folding emphasizes the existence of a single folding pathway consisting of sequential steps and assumes that the majority of interactions that have appeared in folding intermediates will still be present in the native structure. Moreover, this model does not take into account several factors that are involved in assisted protein folding inside cells, such as factors involved in proline isomerization, formation and rearrangement of disulfide bonds (reviewed in Creighton et al., 1996), or occurrence of transient multimeric protein complexes during folding (Pecorari et al., 1996). In this review, the relationship between protein folding *in vitro* and *in vivo* is discussed, using as an example the folding of the hCG- β subunit for which a folding pathway has been established both *in vitro* and in intact cells (Huth et al., 1993).

Role of disulfide bond formation in protein folding and assembly

It has been clear for a long time that the *in vitro* folding of proteins targeted for secretion is facilitated by folding in the presence of microsomal extracts (Goldberger et al., 1963). It is now known that microsomes contain many co-factors and chaperones that foster protein folding (Gething & Sambrook, 1992) as well as the systems to create a favorable redox potential for the formation of disulfide bonds (Lyles & Gilbert, 1991; Braakman et al., 1992; Hwang et al., 1992), which is crucial for the stabilization of tertiary structure of many secreted proteins and for the assembly of a number of multimeric proteins (Seckler & Jaenicke, 1992). For disulfide-containing proteins, it is clear that PDI is a key factor facilitating disulfide bond formation-dependent folding because the rate of stimulation of bovine pancreatic trypsin inhibitor (BPTI) folding by microsomal extracts is en-

hanced to the extent expected by the PDI activity of these extracts (Zapun et al., 1992). Moreover, PDI stimulates the rate of folding of kinetically trapped BPTI molecules *in vitro* by several thousand-fold, but has little effect on disulfide bond formation of BPTI molecules possessing disulfide bonds that form efficiently in the absence of PDI (Weissman & Kim, 1993).

A classic model for protein folding is ribonuclease, which contains four intramolecular disulfide bonds that are required for its enzymatic activity (Anfinsen, 1973). Anfinsen was the first to observe that if a reduced denatured preparation of RNase was left to reoxidize by itself in a test tube in air overnight, a large amount of the original catalytic activity was restored (Anfinsen & Haber, 1961). This simple experiment not only led to the Anfinsen hypothesis that all the information to obtain native structure is present in the primary sequence, but also showed the importance of the role of disulfide bonds because merely removing the urea used as the denaturant while keeping the preparation in an oxygen-free atmosphere did not restore enzymatic activity (reviewed in Richards, 1995).

The pioneering work of Creighton and his colleagues (reviewed in Creighton et al., 1996) employed the formation of intramolecular disulfide bonds as a biochemical probe to study the folding pathway of BPTI. This 58-amino acid protein has three intramolecular disulfide bonds and, when reduced and denatured, its folding pathway can be followed *in vitro* by the reformation of the disulfide bonds. Using alkylation with iodoacetic acid to trap free thiols, Creighton et al. defined a folding pathway involving intermediates that varied in their amount and type of disulfide bonds. They also observed the existence of a significant, albeit mostly transient, population of intermediates containing disulfide bonds not present in the native protein. As pointed out by Weissman and Kim (1992), there are a number of notable features of the BPTI *in vitro* folding pathway that are of particular interest. For one thing, only folding intermediates containing native disulfide bonds are highly populated. Second, the folding of BPTI does not result from the sequential formation of its three (5-55, 30-51, and 14-38) disulfide bonds, but rather is the result of disulfide bond rearrangements. This requirement for BPTI folding to proceed through non-native disulfide-containing species is due to the premature formation of native-like structure. In addition, nearly half of the BPTI molecules recovered are kinetically trapped and unable to fold efficiently to native BPTI. However, these authors also point out that if the pro-region (consisting of 13 amino acids containing a very crucial cysteine residue) is left on the BPTI molecule, refolding of the molecule *in vitro* is many times more rapid than in its absence, and the amount of kinetically trapped material is reduced dramatically. This is a significant finding, because the folding of BPTI in the ER probably proceeds by this latter mechanism. The key aspects of the BPTI folding pathway have been confirmed in several studies (Weissman & Kim, 1992; Goldenberg et al., 1993; Creighton et al., 1996). Taken together, the data indicate that disulfide rearrangements occur and that non-native disulfide bonds are important though transient intermediates in the BPTI folding pathway.

There is also evidence that disulfide bond rearrangement occurs in proteins folding in intact cells. For example, in the intracellular folding pathway of the hCG- β subunit determined by pulse-chase kinetics (Huth et al., 1992a, 1992b) and by site-directed mutagenesis of cysteines involved in disulfide bonds (Bedows et al., 1993, 1994), two of the six disulfide bonds

formed during the kinetic folding pathway of hCG- β are different from those seen in the crystal structure of the native protein (Lapthorn et al., 1994; Wu et al., 1994a). In addition, some of the hCG- β subunit forms generated in cells transfected with β genes mutated at each of the six disulfide pairs do not progress to the native folded form, but are stable and are secreted, whereas other mutated forms are degraded intracellularly (Bedows et al., 1994). These data indicate that un(mis)folded hCG- β subunits may be targeted for secretion or degradation depending on their tertiary structure, which is detected by the presence or absence of particular native disulfide bonds. It should be noted that, although there is some agreement that formation of *intramolecular* disulfide bonds does not determine tertiary folding events, there is general agreement that their formation stabilizes native tertiary structure (Jaenicke, 1991). Formation of *intermolecular* disulfide bonds is involved in the assembly and oligomerization of some multiunit proteins (quaternary structure).

Another model of *in vivo* protein folding that has been studied is influenza hemagglutinin (HA) (Chen et al., 1995). The folding of HA in the ER has also been followed by the formation of intrachain disulfide bonds. Folding of HA starts cotranslationally with some disulfide bonds beginning to form soon after both cysteines that are involved in an S-S pair enter the ER lumen. However, most S-S bond formation in HA occurs after nascent polypeptide bond termination. Chen et al. did not see evidence of formation of aberrant disulfide bonds unless folding was perturbed by inhibitors of glycosylation or glucose trimming.

The importance of the formation of disulfide bonds during protein folding in the ER can be seen by what happens when this is prevented by alteration of the redox potential inside cells. The addition of dithiothreitol (DTT) to the medium of cultured cells expressing HA prevented disulfide bond formation and HA secretion (Tatu et al., 1993). Removal of DTT from the medium allowed proper folding, oligomerization, and secretion of HA. Similarly, in cultured human hepatoma cells, secretion of proteins with intramolecular disulfide bonds was reversibly blocked by treatment of cells with DTT, but secretion of proteins not containing disulfide bonds was not affected (Lodish & Kong, 1993). The role of redox potential and protein disulfide isomerase, the protein that facilitates disulfide bond formation, breakage, and rearrangement in the ER of cells, has been reviewed in detail elsewhere (Gilbert, 1990; Freedman, 1995).

Role of glycosylation in protein folding and assembly

Because many membrane and secretory proteins are glycoproteins, it is important to consider the role of carbohydrates in protein folding, assembly, and secretion. Eukaryotic multicellular organisms utilize oligosaccharides for different purposes. In the ER, high mannose N-linked oligosaccharides are added and partially trimmed. Here they play a role in protein folding and assembly and act as part of the quality control system that the ER has to monitor correct folding and assembly (Helenius, 1994). In the Golgi, N-linked oligosaccharides of glycoproteins are further trimmed and processed, O-linked glycans are added, and oligosaccharides become important for intracellular translocation and targeting of proteins to specific organelles. Once glycoproteins are secreted, oligosaccharides are involved in determining their plasma half-lives and, at the target organ, if the glycoproteins have some hormonal or growth factor type action,

oligosaccharides are important for receptor binding and signal transduction events.

N-linked oligosaccharides of the high-mannose composition are added cotranslationally to the Asn-X-Ser(Thr) consensus sequence of proteins in the ER. One function of N-linked glycans is to facilitate protein folding and conformational maturation. When N-linked chains are eliminated by site-directed mutagenesis of Asn residues in glycosylation consensus sequences or by treatment of cells with agents that block addition of N-linked glycans or their processing, many ER-synthesized glycoproteins misfold, aggregate, and get degraded within the ER (reviewed in Helenius, 1994). There are, however, other glycoproteins that seem to fold and be translocated efficiently without their N-linked glycans. The only rule that seems to emerge here is that glycoproteins, often larger ones, that fold slowly in the first place have even more trouble folding if their N-linked glycans are missing.

There is a quality control point in protein folding of glycoproteins in the ER that involves the chaperone calnexin. Calnexin acts as a retention factor for high-mannose oligosaccharide-containing glycoproteins terminating with one glucose residue (Hebert et al., 1995). The processing of high-mannose N-linked glycans is initiated in the ER by two glucosidases that trim terminal glucose residues. Incompletely folded proteins that retain one terminal glucose residue are substrates for binding to calnexin, an ER membrane-associated chaperone that binds and retains its substrates in the ER. As folding progresses, calnexin releases its substrate to subsequent chaperones in the folding cascade that facilitate formation of a fully folded native protein. Release of glycoproteins from calnexin requires trimming of the terminal glucose residue by glucosidase II (Hebert et al., 1995). Incompletely folded or misfolded proteins may be released by calnexin and re-glucosylated by UDP-glucose:glycoprotein glucosyltransferase to be recycled back through the folding pathway, thus providing a salvage pathway. Not all glycoproteins appear to use this scheme and it is clear that there is a redundancy of folding factors in the ER that can foster protein folding.

Role of molecular chaperones

The role of molecular chaperones in protein folding, assembly, and intracellular translocation is a burgeoning area of research and has been the subject of a number of recent reviews (Jakob & Buchner, 1994; Landry & Gierasch, 1994; Hendrick & Hartl, 1995; Ellis & Hartl, 1996). ER chaperones play a key role in protein folding and quality control. Cytosol chaperones play a key role in folding, transport, and biological activity of a number of proteins targeted for transport to specific organelles such as the nucleus and mitochondria. One example of this is the role of members of the heat-shock proteins of the Hsp70 and Hsp90 families in steroid hormone receptor binding and translocation (Pratt, 1993).

Important members of the ER family of chaperones include BiP, characterized originally as an immunoglobulin binding protein, hence the name (Bole et al., 1986), GRp (or ERp) 72 (Nigam et al., 1994), GRp (or ERp) 94 (Melnick et al., 1992), calnexin (Ou et al., 1993), and calreticulin (Nauseef et al., 1995). Other chaperones continue to be discovered and characterized, such as the cytosol chaperones Hsp40 and Hip, a co-chaperone

involved in the Hsc70/Hsp40 reaction cycle (Hohfeld et al., 1995). The enzyme-like cofactors protein disulfide isomerase (Noiva & Lennarz, 1992) and peptidyl prolyl *cis/trans* isomerase (Schmid, 1993), which catalyzes the isomerization of *trans*- to *cis*-proline, are usually considered ER chaperones as well.

Chaperones appear to be members of a "bucket brigade" that bind to folding intermediates that are in various stages of folding and then pass them on to the next chaperone or chaperone complex in the cascade, eventually releasing a competent native protein (Hammond & Helenius, 1994; Melnick et al., 1994; Kim & Arvan, 1995). Binding usually involves interaction of the surface hydrophobic sites on unfolded proteins and release often involves ATP hydrolysis. Although no specific consensus sequences for chaperone binding have been determined, binding of BiP by folding intermediates, as an example, is favored by seven-residue amino acid sequences with aliphatic amino acid residues in alternating positions for β -sheets or every three to four residues for α -helices in the peptide (Flynn et al., 1991). These are the sort of sequences that would normally be on the inside of native proteins, providing a way for chaperones to discriminate between folded and unfolded proteins.

What happens to misfolded proteins

It is generally thought that misfolded proteins remain in the ER and are degraded there without being secreted. We now know that there is at least one exception to this [i.e., the hCG- β subunit (see below)], and there are most likely others. Nevertheless, there are a number of examples where protein misfolding leads to protein accumulation in the ER and degradation. Temperature-sensitive mutants of vesicular stomatitis virus G protein fail to fold at the nonpermissive temperature; disulfide bonds are not correctly formed; the G-protein is tightly bound to BiP; and the protein is not translocated to the cell surface (deSilva et al., 1990). When the temperature is lowered to the permissive temperature, the G-protein is properly folded and transported to the cell surface. Unfolded H2b asialoglycoprotein receptor subunit polypeptides are also degraded selectively in the ER (Wikstrom & Lodish, 1993). Unassembled subunits of the T-cell antigen receptor fail to reach the Golgi and are degraded in the ER in a manner dependent on the redox potential (Young et al., 1993).

Some molecular chaperones appear to be involved in targeting irreversibly misfolded proteins for degradation in the ER. BiP is one of these. For example, immunoglobulin light chains that are slowly folding and retained in the ER of cultured mouse cells are quantitatively bound to BiP as partially disulfide bonded forms, and then degraded, whereas light chains that are more rapidly folded and secreted only transiently interact with BiP (Knittler et al., 1995).

Degradation of un(mis)folded proteins can clearly take place in the ER without transport from the ER. This has been shown, for example, by comparing the degradation of T-cell antigen receptor (TCR) subunits in intact versus permeabilized cells. TCR subunits are degraded with the same kinetics in cells with intact membranes as in those in which membrane pores are induced by streptolysin O (Stafford & Bonifacino, 1991). Moreover, it was shown by immunofluorescence and cell fractionation studies that the proteins do not leave the ER intact and that the selective degradation of TCR subunits can be carried out by an isolated ER fraction.

Disease states related to protein misfolding

A number of human disease states are related to protein folding defects. Two classic examples are cystic fibrosis (CF) and α_1 -antitrypsin deficiency. In one variant of CF, the most common one—deletion of phenylalanine at residue 508 (CFTR Δ F508), the cystic fibrosis transmembrane conductance regulator (CFTR) is not correctly processed, is retained in the ER, and does not reach the plasma membrane in normal amounts (Denning et al., 1992). The emphysema-related Z variant of α_1 -antitrypsin, which is inherited in 1/1,600 live births, results from a Glu 342 $\rightarrow$ Lys substitution that prevents normal folding of the enzyme and causes its retention in the ER of the liver, where much of it is degraded and the remainder forms insoluble intracellular inclusions (Wu et al., 1994b). This has now been clearly shown to result from a folding defect in the Z variant form of α_1 -antitrypsin (Yu et al., 1995).

There are also other examples. Hereditary cystatin C amyloid angiopathy is a dominantly inherited disease characterized by dementia, paralysis, and death from cerebral hemorrhage in early adult life. There is a disease-associated Leu 68 $\rightarrow$ Gln mutation in a cysteine proteinase inhibitor, cystatin C, that causes the cystatin C to aggregate as amyloid in patients' tissues (Abrahamson & Grubb, 1994).

Aspartylglycosaminuria is a common glycoprotein degradation syndrome seen in the Finnish population (1/4,000 frequency). It is characterized by autosomal recessive inheritance, psychomotor retardation, and accumulation of large amounts of glycoasparagines in tissues and body fluids. The biochemical defect is a Cys 163 $\rightarrow$ Ser mutation that disrupts a key disulfide bond, resulting in loss of catalytic activity, defective subunit processing, and loss of lysosomal targeting of glycosylasparaginase (McCormack et al., 1995).

There are five dominantly inherited neurodegenerative diseases that have genetic defects involving CAG trinucleotide repeats that tend to lengthen in successive generations, especially via male transmission. These are Huntington's chorea, spinal/bulbar muscular atrophy, spinocerebellar ataxia type I, dentatorubral-pallidoluysian atrophy, and Machado-Joseph disease. This defect results in abnormally expanded stretches of polyglutamine in affected proteins. Based on model polypeptides containing such repeats, it has been found that glutamine repeats can form β -pleated sheets or type I- β turns that cause the polypeptides to aggregate (Stott et al., 1995). The data suggest that pathologic conditions may occur when expanded glutamine repeats cause proteins to aggregate with themselves or other proteins.

It is also likely that certain forms of Alzheimer's disease result from protein folding defects. For example, a single mutation of Val 18 $\rightarrow$ Ala in the N-terminal domain of amyloid- β peptide induces a significant increase in α -helical content, which decreases formation of amyloid fibrils (Soto et al., 1995). In hereditary Dutch cerebral hemorrhagic disease with amyloidosis, a form of Alzheimer's, there is a Glu 22 $\rightarrow$ Gln substitution that decreases the α -helical content of the N-terminal domain of amyloid- β peptide and increases amyloid formation (Soto et al., 1995).

It seems likely that other diseases will be shown to result from protein folding defects as we learn more about protein folding in intact cells. Cancer may be one of these diseases. In the case of the hCG- β subunit, discussed as a model below, there is evi-

dence that un(mis)folded forms of the β subunit circulate in the plasma of patients with gynecologic malignancies (K. Duffy & R. Ruddon, unpubl. obs.). The inability of cancer cells to deposit a normal extracellular matrix may be another example (Frenette et al., 1988).

hCG- β subunit as an *in vivo* model of secretory protein folding

One of the few, and perhaps the only mammalian, protein for which a folding pathway in intact cells has been established (Lodish & Kong, 1993) is the β subunit of the glycoprotein hormone human chorionic gonadotropin (hCG) (Bedows et al., 1992, 1993; Huth et al., 1992a, 1992b). As such, it has served as a useful model for *in vivo* protein folding. Because it is a secretory glycoprotein with six intramolecular disulfide bonds and must assemble with its partner 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 (S-S) bonds as an index of conformational changes during protein folding, the intracellular folding pathway of the β subunit of hCG has been determined (Bedows et al., 1992, 1993; Huth et al., 1992a, 1992b). Three folding intermediates of β were purified from [35 S]cysteine-labeled JAR choriocarcinoma cells or β -gene transfected CHO cells by immunoprecipitation and by reverse-phase HPLC. To identify unformed disulfide bonds, nonreduced, carboxymethylated folding intermediates were treated with trypsin to liberate nondisulfide-bound, [35 S]cysteine-containing peptides from the disulfide-linked peptides. Released peptides were purified by HPLC and identified by amino acid sequencing. The amount of a peptide that was released indicated the extent of an unformed S-S bond involving the cysteine in that peptide. Of the six disulfide bonds in hCG- β , the S-S bonds between cysteines 34-88 and 38-57 form first. The rate-limiting event of folding involves the formation of the S-S bonds between cysteines 9-90 and 23-72. The disulfide bond 93-100, the formation of which appears to be necessary for assembly with the α subunit of the hCG heterodimer, forms next. Finally, the disulfide bond 26-110 forms after assembly with α , demonstrating that completion of folding of the C-terminus in the β subunit occurs after assembly with α . This correlates with what is now known about the crystal structure of native hCG in that the 26-110 disulfide bond of β forms a "seat belt" around the α subunit after assembly (Lapthorn et al., 1994; Wu et al., 1994a). The intracellular kinetic folding pathway (Bedows et al., 1992, 1993; Huth et al., 1992a, 1992b) identified by the precursor-product relationships in these pulse-chase studies is (numbers above the arrows represent the disulfide bonds formed at each step):

The role of individual S-S bonds in the formation of the folding intermediates of the hCG- β subunit, identified by the pulse-chase kinetics, was tested by employing CHO cells transfected with mutated hCG- β genes in which the Cys residues required

for the formation of S-S bonds were replaced by Ala (Bedows et al., 1993, 1994). When the Cys residues required for the 34-88, 38-57, or 9-90 S-S bonds to form were substituted with Ala, folding of hCG- β beyond the earliest detectable folding intermediate (p β 1-early) was inhibited. When the Cys residues forming S-S bond 23-72 were deleted, p β 1-early was converted into a second folding intermediate (p β 1-late) with wild-type kinetics, and some conversion to the next intermediate (p β 2-free) occurred. However, it was not as stable as wild-type p β 2. About 25% of the p β 2 containing the β 23-72 mutation assembled with α , and the $\alpha\beta$ dimer thus formed had biological activity in a receptor-binding assay. When either of the final two S-S bonds (the carboxyl-terminal 93-100 or 26-110 bonds) were removed, conversion of p β 1-late to p β 2-free was detected, but formation of $\alpha\beta$ dimer was not observed. These data support the hypothesis that individual S-S bonds are involved in discrete steps in the hCG- β folding and assembly pathway.

Although some purified proteins will refold and assemble *in vitro*, it was not previously known if cellular factors change the mechanisms of these processes. Based on the gel migration of folding intermediates, the kinetic relationships between these intermediates, and the order of formation of six disulfide bonds, it was found that the *in vitro* folding pathway of the hCG- β subunit is indistinguishable from the intracellular folding pathway (Huth et al., 1993). The same rate-limiting event was found in both folding environments; however, the $t_{1/2}$ for this step *in vivo* is 5 min, whereas *in vitro* the $t_{1/2}$ is ≥ 80 min. Protein disulfide isomerase (PDI) increased the *in vitro* rate of this event ($t_{1/2} = 25$ min) without changing the order of disulfide bond formation. It is possible that the *in vitro* rate of folding of β in the presence of PDI could approach the intracellular rate if the amount of PDI used was closer to that reported to be present in the ER, estimated to be in the millimolar range (Lyles & Gilbert, 1991).

PDI also catalyzed the *in vitro* rate of assembly of hCG subunits. In intact cells, assembly of the $\alpha\beta$ heterodimer occurs before the final intramolecular disulfide bond (26-110) is formed. *In vitro*, assembly is increased after reduction of the two carboxyl-terminal S-S bonds (93-100 and 26-110) of hCG- β by PDI (Huth et al., 1993). These results strongly suggest that, both in intact cells and *in vitro*, partially unfolded hCG- β is more assembly-competent than is fully folded hCG- β .

The role of N-linked oligosaccharide chains in intracellular folding of the hCG- β subunit was determined by examining the kinetics of folding in CHO cells transfected with wild-type or mutant hCG- β genes lacking one or both of the asparagine glycosylation sites (Feng et al., 1995b). The inefficient folding of hCG- β lacking both N-linked glycans correlated with the slow formation of the last three disulfide bonds (i.e., disulfides 23-72, 93-100, and 26-110) to form in the hCG- β folding pathway. Unglycosylated hCG- β was slowly secreted from CHO cells, and β subunit folding intermediates retained in cells for more than 5 hours were degraded into a smaller hCG- β fragment. However, coexpression of the hCG- α gene enhanced folding and formation of disulfide bonds 23-72, 93-100, and 26-110 of hCG- β lacking N-linked glycans. In addition, the molecular chaperones BiP, ERp72, and ERp94, but not calnexin, were found in a stable complex with unglycosylated, unfolded hCG- β and may be involved in the folding of this β form (Feng et al., 1995b). These data indicate that N-linked oligosaccharides assist hCG- β subunit folding by facilitating disulfide bond formation, perhaps

by increasing the stability and solubility of the native structure that fosters disulfide formation.

It was also found that the formation of stable complexes of hCG- β with ER chaperones occurs in several CHO cell lines transfected with the wild-type β gene or a variety of slow-folding mutants of hCG- β (Feng et al., 1996). The amount of hCG- β -chaperone complex correlated with the rate and extent of hCG- β subunit folding. The slower folding mutants formed a greater detectable amount of chaperone complex, but the non-folding 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. The hCG- β -chaperone complexes involve the formation of intermolecular disulfide bonds because separation of hCG- β folding intermediates from chaperones occurred only under a combination of denaturing and reducing conditions, but not under denaturing conditions alone. This indicates that disulfide bond formation is an essential characteristic of the molecular chaperone- β interaction.

To determine if there was a differential role of asparagine-linked oligosaccharide chains in the protein folding versus assembly components of the hCG- β secretory pathway, the hCG- β in vitro folding and assembly assays were used to analyze how the hCG- β subunit devoid of one or two N-linked glycans folds and assembles under different conditions in vitro (Feng et al., 1995a). Using the substrates mutated at the N-linked glycosylation sites from CHO cells, it was found that only 10% of p β 1 missing two N-linked glycans and 40% of p β 1 missing just the Asn 30-linked glycan were converted to the corresponding p β 2, respectively, whereas the p β 1 missing the Asn 13-linked glycan folded to p β 2 with the same rate as wild-type β . These data demonstrate that at least one N-linked glycan is required for efficient folding of hCG- β and that the Asn 30-linked glycan is more important than the Asn 13-linked glycan for hCG- β folding. It also was shown that the composition of N-linked glycans of hCG-p β 1 did not change protein folding, because hCG- β substrates with high-mannose oligosaccharides folded as efficiently as β substrates containing sialylated, complex oligosaccharides. Moreover, assembly of the already folded, assembly-competent folding intermediate, p β 2, was not affected by removal of one or both of the N-linked glycans of the β subunit, indicating that once β is folded, its assembly with α is not affected by the presence or absence of N-linked glycans.

It is also of interest that certain un(mis)folded forms of hCG- β are secreted rather than retained and degraded in the ER of intact cells. Site-directed mutagenesis of every pair of Cys residues involved in the formation of each S-S bond was again used to examine the roles that S-S bonds play in β subunit secretion (Bedows et al., 1994). Tryptic maps of secreted hCG- β showed that only one S-S bond formed in all S-S bond mutants that failed to fold from the earliest detectable β folding intermediate, p β 1, into the second major intermediate, p β 2 (C34A-C88A, C38A-C57A, or C9A-C90A mutants), whereas all five remaining S-S bonds (other than the one deleted) formed in mutants when p β 1 $\rightarrow$ p β 2 conversion occurred (C23A-C72A, C93A-C100A, or C26A-C110A mutants). Nonreducing SDS-PAGE showed that β multimers were secreted from cells expressing S-S bond mutations where the folding of p β 1 $\rightarrow$ p β 2 was blocked. However, for mutations where p β 1 $\rightarrow$ p β 2 conversion was efficient, β monomers rather than multimers were secreted. For all cell lines studied, secreted hCG- β migrated as monomeric β during reduc-

ing SDS-PAGE, indicating that hCG- β multimers formed via intermolecular crosslinking of unpaired thiols. Tryptic maps of hCG- β isolated from mutants lacking the 34-88 bond, where $>80\%$ turnover occurs, showed that only the 38-57 S-S bond formed. β subunits lacking the 9-90 linkage also have only S-S bond 38-57 formed, but $<10\%$ turnover of C9A-C90A hCG- β occurs. Thus, conformational differences between partially folded or misfolded β subunits may determine whether hCG- β is degraded or is translocated and secreted. This, in turn, may be determined by what cadre of chaperones bind to the various un(mis)folded hCG- β forms.

Structural model of hCG-folding

The kinetic pathway defined in terms of the disulfides present at various stages of folding has limitations, because it cannot directly reflect development of secondary, tertiary, and quaternary structures. The kinetic pathway of hCG- β folding (Huth et al., 1992a, 1992b) was established before X-ray analysis of hCG was completed (Lapthorn et al., 1994; Wu et al., 1994a). X-ray analysis of hCG showed that the three-dimensional structures of both hCG- α and hCG- β subunits have pronounced similarities. In the hCG heterodimer, α and β subunits are arranged in an antiparallel fashion as illustrated in the schematic model shown in Figure 1. The subunits have elongated structures,

Fig. 1. Schematic diagram of hCG heterodimer structure. The α subunit is shown in green and the β subunit in blue. Heavy solid bars represent β -strands. Locations of the cysteines involved in disulfide bridges (red lines) are shown by circles. N, amino terminus; C, carboxyl terminus.

formed from three hairpin-like fragments: H1- α , H2- α , and H3- α in α ; H1- β , H2- β , and H3- β in β . A distinguishing feature of each subunit is a cystine knot. The knot of α is formed by a ring, consisting of the residues 28–32 and 82–84 bridged by two S-S bonds (28–82 and 32–84) through which a third disulfide bridge, 10–60, penetrates. An analogous knot of β is formed by a ring (residues 34–38 and 88–90, bridged by S-S bonds 34–88 and 38–90) through which the 9–57 S-S bridge penetrates. In α , there are two other disulfide bridges, 7–31 and 59–87, that stabilize H1- α and H3- α hairpins. In β , there are three other disulfide bridges, 23–72, 93–100 and 26–110, in addition to the cystine knot.

Comparison of disulfide bonds observed in the crystal structure of the native protein with disulfide bonds formed in the earliest detected intermediates, p β 1 and p β 2, shows that two of the six disulfide bonds formed in the folding intermediates are different from those seen in the crystal structure. The intracellular folding pathway of the hCG- β subunit determined by pulse-chase kinetics (Huth et al., 1992a, 1992b) and by site-directed mutagenesis of cysteines involved in disulfide bonds (Bedows et al., 1993, 1994), indicates that two of the disulfide bonds (38–57 and 9–90) are different from those (9–57 and 38–90) seen in the crystal structure. Thus, it appears that to form native structure, rearrangements of early formed disulfide bridges must take place.

A structural model of hCG- β folding showing development of secondary and tertiary structures of hCG- β has been developed by our laboratory (Fig. 2). One may postulate that folding of the hCG- β nascent polypeptide chain starts with formation of two hairpin-like structures, H1- β and H3- β (p β 0-early, see Fig. 2A). According to X-ray data, H1- β and H3- β are antiparallel β structures and probably form in milliseconds. This structure would be stabilized mainly by hydrogen bonding within its polypeptide backbone and by interactions within two hydrophobic clusters. One cluster belongs to the H1- β fragment and includes residues Leu 16, Val 18, Ile 27, and Val 29. The other cluster, including residues Ile 67, Leu 69, Val 80, and Tyr 82, belongs to H3- β .

In the next step, formation of p β 0-late (Fig. 2B), these two clusters collapse hydrophobically and the hydrophobic core derived from side chains of Leu 16, Val 18, Ile 27, Val 29, Ile 67, Leu 69, Val 80, and Tyr 82 residues would form. In p β 0-late, two hairpin-like structures, H1- β and H3- β , come into close proximity, giving a general shape to the β subunit. The shape is similar to that of native hCG- β . This intermediate folding transition may be similar to the “molten globule,” which represents a form with significant secondary structure, but little tertiary structure (Ptitsyn, 1991).

In this state, the Cys 34 and Cys 88 residues come into close proximity, giving them the possibility of forming an S-S bridge (p β 1-early, Fig. 2C). The pulse-chase data indicate that the S-S bond 34–88 is formed within 2 min intracellularly (Huth et al., 1992a). The S-S bridge 38–57 is formed within 3 min (p β 1-late, Fig. 2D). These two S-S bonds help to stabilize the spatial proximity of H1- β and H3- β . Additional stabilization comes from formation of the 9–90 and 23–72 S-S bridges that restrict flexibility of the H1- β and H3- β loops (p β 2-early, Fig. 2E). This permits the rearrangement of the 38–57 and 9–90 S-S bonds to create the cystine knot, 9–57 and 38–90, found in the hCG X-ray structure (p β 2-rearranged, Fig. 2F). Because in the cystine knot structure the S-S bond 9–57 crosses through the ring of two other S-S

bridges, 34–88 and 38–90, it is unlikely that 9–57 could form after the 34–88 and 38–90 bonds. Given the fact that the bridge 34–88 is formed at the p β 1 stage and there is no evidence of its rearrangement, creation of the cystine knot requires that 9–57 be formed before the 38–90 bridge. Presumably, creation of the cystine knot occurs between 4 and 10 min, which is the time during which folded p β 2 subunit appears intracellularly (Huth et al., 1992a).

With formation of the 23–72 S-S bond and the cystine knot, the core structure of hCG- β becomes very stable. However, C-terminal residues (93–145) of the β subunit are still relatively flexible. This flexibility is probably a major obstacle for α - β heterodimerization. Only after formation of the S-S bridge 93–100 (p β 2-free, Fig. 2G) does the surface of hCG- β become complementary to the hCG- α surface ($t_{1/2}$ of 8–10 min). This allows for the formation of a α - β heterodimer in which α and β subunits are arranged in an antiparallel fashion. Assembly of the α and β subunits is stabilized by the formation of a “seat belt” that is a part of the C-terminus of hCG- β (residues 93–110) (Lapthorn et al., 1994; Wu et al., 1994a). The seat belt envelopes the H2- α loop of the α subunit and clasps it to the H1- β loop of the β subunit (see Fig. 2H). The final closing of the 26–110 bridge locks the seat belt and secures the $\alpha\beta$ dimer preventing disassembly. Most $\alpha\beta$ dimers are formed with $t_{1/2}$ of 15–20 min intracellularly.

Summary

Studies of the folding pathway of the hCG- β subunit have revealed some important information about how glycoproteins fold, assemble, and are secreted by mammalian cells. A number of salient features can be described, based on these studies.

1. The order of disulfide bond formation of the hCG- β subunit is the same intracellularly as in vitro, but the $t_{1/2}$ for the rate-limiting step (p β 1 $\rightarrow$ p β 2) is 4–5 min in vivo and 80 min in vitro under optimized in vitro redox conditions.
2. Addition of protein disulfide isomerase to the in vitro folding reaction decreases the $t_{1/2}$ of the rate-limiting step from 80 to 25 min, but does not alter the order of formation of the disulfide bonds.
3. Studies of mutants of hCG- β in which each of the six intramolecular disulfide bonds has been selectively eliminated by site-directed mutagenesis demonstrate that discrete disulfide bonds are formed in each detectable intermediate in the folding pathway.
4. The asparagine (N)-linked oligosaccharides play their most important role in the folding component of the folding and assembly pathway of hCG- β .
5. The N-linked oligosaccharides assist hCG- β subunit folding by facilitating disulfide bond formation, perhaps by increasing the stability and solubility of the native structure that fosters disulfide formation.
6. hCG- β folding intermediates form stable complexes with a number of ER chaperones, including BiP, ERp 72, and GRp 94. These complexes are linked by intermolecular disulfide bonds and ultimately lead to production of folded, secreted hCG- β , suggesting that these complexes are functional intermediates in a productive folding pathway.
7. The intracellular kinetic folding pathway of hCG- β determined by pulse-chase labeling indicates that two of the six

Fig. 2. The hCGβ folding pathway. **A,B:** Two earlier (hypothetical) intermediates. Amino acids important for the formation of these intermediates are shown as ovals. **C,D:** First intermediates, pβ1-early and pβ1-late, that have been detected by kinetic studies. **E:** Second intermediate, pβ2-early, that has been detected by kinetic studies. **F:** Hypothetical intermediate, pβ2-rearranged, that is needed to obtain the cystine knot. **G:** Intermediate, pβ2 free, that is ready to combine with α subunit. **H:** Mature β-combined state in the αβ heterodimer.

intramolecular disulfide bonds formed during folding are different from those observed in the crystal structure of the native hormone, suggesting that non-native disulfide bonds form and are rearranged during intracellular folding. Although this has been observed for in vitro folding of some proteins, such as BPTI, this is the first indication that this also occurs in vivo.

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