THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 34, Issue of August 20, pp. 35426–35436, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

I. THE GLYCOSYLATED END OF HUMAN α-SUBUNIT LOOP 2 IS THREADED THROUGH A β-SUBUNIT HOLE*

Received for publication, March 18, 2004, and in revised form, May 24, 2004
Published, JBC Papers in Press, May 25, 2004, DOI 10.1074/jbc.M403052200

Yongna Xing, Rebecca V. Myers, Donghui Cao, Win Lin, Mei Jiang, Michael P. Bernard, and William R. Moyle‡

From the Department of Obstetrics and Gynecology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson (Rutgers) Medical School, Piscataway, New Jersey 08854

Glycoprotein hormone heterodimers are stabilized by their unusual structures in which a glycosylated loop of the α-subunit straddles a hole in the β-subunit. This hole is formed when a cysteine at the end of a β-subunit strand known as the "seatbelt" becomes "latched" by a disulfide to a cysteine in the β-subunit core. The heterodimer is stabilized in part by the difficulty of threading the glycosylated end of the α-subunit loop 2 through this hole, a phenomenon required for subunit dissociation. Subunit combination *in vitro*, which occurs by the reverse process, can be accelerated by removing the α-subunit oligosaccharide. In cells, heterodimer assembly was thought to occur primarily by a mechanism in which the seatbelt is wrapped around the α-subunit after the subunits dock. Here we show that this "wraparound" process can be used to assemble disulfide cross-linked human choriogonadotropin analogs that contain an additional α-subunit cysteine, but only if the normal β-subunit latch site has been removed. Normally, the seatbelt is latched before the subunits dock and assembly is completed when the glycosylated end of α-subunit loop 2 is threaded beneath the seatbelt. The unexpected finding that most assembly of human choriogonadotropin, human follitropin, and human thyrotropin heterodimers occurs in this fashion, indicates that threading may be an important phenomenon during protein folding and macromolecule assembly in the endoplasmic reticulum. We suggest that the unusual structures of the glycoprotein hormones makes them useful for identifying factors that influence this process in living cells.

The glycoprotein hormones are essential for vertebrate reproduction and thyroid function. Each of the four human hormones is composed of non-covalently bound α- and β-subunits that are divided into three large loops (α1, α2, α3; β1, β2, β3)[1] by a cystine knot (1–3). The β-subunit also contains 20 additional residues that form a strand commonly known as the "seatbelt" because of its topology and its role in stabilizing the heterodimer (1). The seatbelt begins at the β-subunit cystine knot and its carboxyl-terminal cysteine is "latched" by a disulfide to a cysteine in loop β1. This creates a hole in the β-subunit that is bordered on one side by the core of the β-subunit and on the other side by the seatbelt. The α-subunit straddles this hole such that the glycosylated end of loop α2 must pass beneath the seatbelt through the β-subunit hole for the heterodimer to dissociate. This contributes to the stability of the heterodimer, which dissociates at low pH or in high concentrations of urea (4), but not in the presence of ionic detergents such as 0.1% sodium dodecyl sulfate. If the seatbelt were to be latched before the subunits combine, the glycosylated end of loop α2 would also need to pass through the β-subunit during heterodimer assembly. This would impede assembly, a notion supported by the finding that removal of this oligosaccharide accelerates assembly *in vitro* substantially (5), a process that occurs by a threading mechanism (6).

Pulse-chase analyses of hCG assembly in cells led to the suggestion that the seatbelt remains unlatched until after the subunits dock with one another (7). In this pathway, which we term "wraparound" (Fig. 1, *upper pathway*), formation of the seatbelt latch disulfide is the final step in heterodimer assembly and occurs after the seatbelt has been wrapped around loop α2. This pathway circumvents the need for the glycosylated end of loop α2 to pass through the hole in the β-subunit and explains the abilities of cells to make cross-linked hormone analogs in which the seatbelt is latched to cysteines added to the α-subunit (8). Assembly of glycoprotein hormone heterodimers can occur by a threading pathway *in vitro* at high subunit concentrations (6). Before heterodimer assembly begins in the threading pathway, the seatbelt is latched to a cysteine in loop β1 (Fig. 1, *lower pathway*).

Studies described here were initiated to learn if threading has a role in the intracellular assembly of the glycoprotein hormones using a strategy that circumvents the need for pulse-chase analysis, the most common approach to studying protein folding in cells. Pulse-chase methods require labeling, isolating, and characterizing partially folded intermediates. Important intermediates such as heterodimers that have not yet latched their seatbelts or in which loop α2 has not been threaded beneath the seatbelt are unstable and difficult, if not impossible to isolate. As shown here based on the abilities of β-subunit analogs to compete for formation of cross-linked and non-cross-linked heterodimers, cells can assemble hCG, hFSH, and hTSH by a threading mechanism. Indeed, it appears as if these human glycoproteins are assembled in the ER primarily by a threading route and that the wraparound pathway is used sparingly, if at all.

EXPERIMENTAL PROCEDURES

Pure recombinant hCG was obtained from Dr. Robert Campbell (Serono Reproductive Biology Institute, Rockland, MA). Constructs

* This work was supported by NICHD National Institutes of Health Grants HD14907 and HD38547. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 732-235-4224; Fax: 732-235-4225; E-mail: moyle@umdnj.edu.

[1] The abbreviations used are: α1, α-subunit loop 1; α2, α-subunit loop 2; α3, α-subunit loop 3; β1, β-subunit loop 1; β2, β-subunit loop 2; β3, β-subunit loop 3; ER, endoplasmic reticulum; hCG, human choriogonadotropin; hFSH, human follitropin; hTSH, human thyrotropin; hCGβ, hCG β-subunit; abbreviations of the analogs used in these studies are described in Fig. 2.

This paper is available on line at http://www.jbc.org

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

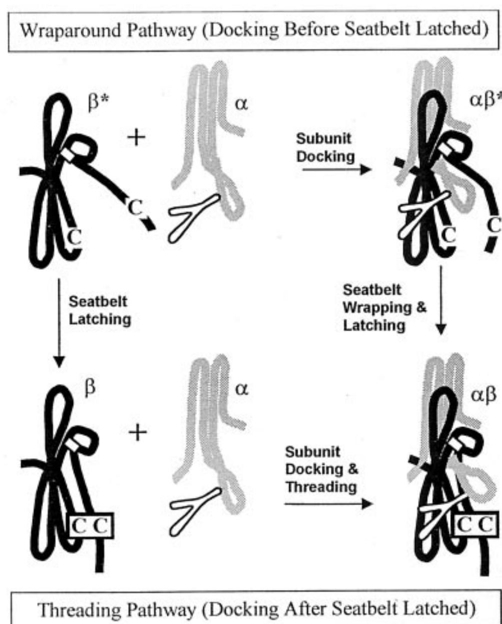

FIG. 1. **Pathways of glycoprotein hormone assembly.** During assembly that occurs by the wraparound pathway (7, 8), the subunits dock before the seatbelt is latched (*upper pathway*); formation of the heterodimer is completed when the seatbelt is wrapped around loop α2 and the latch disulfide is formed (*right vertical pathway*). In the threading pathway (6), the seatbelt latch disulfide forms before the subunits dock (*left vertical pathway*); assembly of the heterodimer is completed when loop α2 and its attached oligosaccharide traverse the hole in the β-subunit beneath the seatbelt (*lower pathway*). Key: α-subunit, *light gray*; β-subunit, *black*; *small rectangle*, disulfide that stabilizes the small loop within the seatbelt; *large rectangle*, seatbelt latch disulfide; *C*, β-subunit cysteines in loop 1 and at the end of the seatbelt; *open y-shaped* figure on α-subunit loop 2, loop α2 oligosaccharide. For simplicity, the remaining oligosaccharides are not depicted.

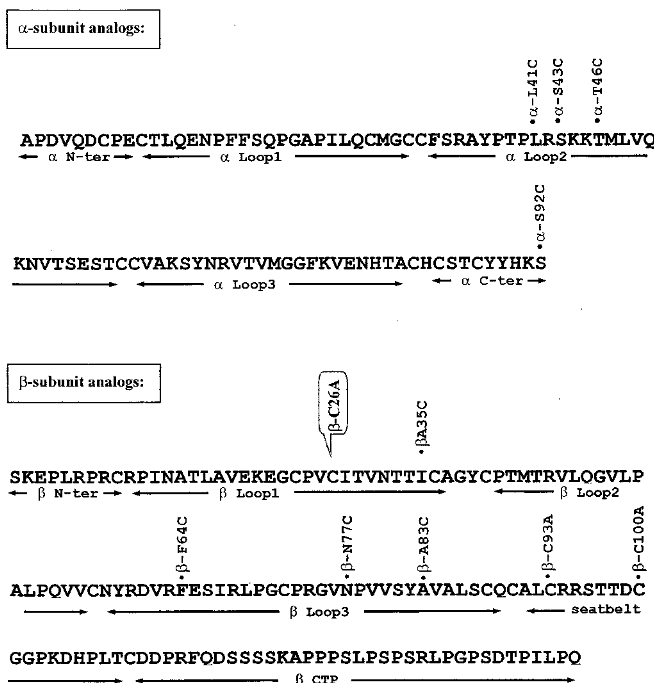

FIG. 2. **Sequences of hCG α- and β-subunit analogs used in this study.** The linear amino acid sequences of each hCG subunit are shown in single letter code. The locations of mutations used in these studies are shown in standard nomenclature. In some cases two mutations were present in a single protein and these are identified by the name of the subunit (*i.e.* hCGβ) and the mutations (*i.e.* -C26A,N77C). Several analogs contained four additional residues at the carboxyl-terminal end of the β-subunit shown as KDEL. The presence of these residues slowed secretion of the heterodimer, presumably because it caused it to be retained in the ER (18). The presence of this sequence is identified by the term -KDEL appended to the name of the analog. *Lines under* the amino acid sequence refer to its position in the protein.

used to express hCG in cultured cells were produced by polymerase chain reaction and cassette mutagenesis (9) and were sequenced prior to use. Analogs used in this study are listed in Fig. 2 and are readily identified by their names. Thus, α-L41C is the natural human α-subunit having a codon for cysteine in place of that for αLeu^{41}. Proteins were produced by transfecting the constructs into COS-7 cells obtained from the ATCC (Bethesda, MD) using a calcium phosphate procedure (10). The amounts of α- and β-subunit constructs used were 20 and 10 μg/10-cm culture plates, respectively. Secreted analogs were harvested 3 days after transfection and analyzed in monoclonal antibody sandwich immunoassays (11) employing antibodies A113, B111, B112, B404, B603, and B806, obtained from Dr. William Munroe (Hybritech Inc., San Diego, CA, a subsidiary of Beckman Coulter, Inc.), B101 was obtained from Dr. Robert Canfield (Columbia University, New York), B122 was obtained from Dr. Robert Campbell, and B110 was produced as described (12). The relative binding sites of these conformation-dependent antibodies have been determined and are indicated in Fig. 3. This figure also illustrates the locations of several residues discussed in this study. A113 recognizes a conformation-dependent α-subunit epitope in the heterodimer. B101 recognizes a conformation-dependent epitope in loop β2 of hCG and the uncombined β-subunit. B110 and B112 recognize conformation-dependent epitopes formed when loops β1 and β3 are adjacent in hCG and the uncombined β-subunit. B112 binds hCG and analogs in which βAsn^{77} is replaced by cysteine but not by aspartic acid or analogs in which a cysteine substitution participates in a disulfide bond. B111 recognizes a conformation-dependent epitope formed when βCys^{110} at the end of the hCG seatbelt is latched to βCys^{26} in the heterodimer and the uncombined β-subunit. B111 can also recognize analogs of hCG in which βCys^{26} and βCys^{110} are converted to alanine provided the heterodimer is stabilized in another fashion, such as fusing the NH_2-terminal end of the α-subunit to the COOH-terminal end of the β-subunit (8). This indicates that B111 does not recognize the βCys^{26}-βCys^{110} disulfide *per se*. B111 does not recognize hCG analogs in which the seatbelt is latched to any other residue other than βCys^{110}, however, or when residues near βCys^{110} are derived from hLH, hFSH, or hTSH. B603 and B806 recognize loops β1 and β3 in the β-subunits of hFSH and hTSH, respectively, but the binding sites of these antibodies have not been well characterized. None of the antibodies used in these studies recognize the β-subunit prior to formation of the cystine knot, a key step in formation of the β-subunit core.

Antibodies used for detection were radioiodinated to a specific activity of ~50 μCi/μg using IODO-GEN (Pierce) as described (13). The acid stability of the heterodimers was tested in 0.4-ml samples by reducing the pH to 2 by addition of microliter aliquots of 2 M HCl, while monitoring the pH, and incubating acidified samples 30 min at 37 °C, readjusting the pH to 7.5 by addition of sufficient microliter aliquots of a mixture of 10 N NaOH, 1 M Tris buffer (pH 7.5) (1:2), and then quantifying them by sandwich immunoassay (11). Material that contained the ER retention signal was isolated from the cells 1 or 2 days after transfection by scraping them from the culture dishes and solubilizing them in 10 mM sodium phosphate buffer (pH 7.5) containing 140 mM KCl, 20 mM EDTA, 1 μM leupeptin, 1.5 μM pepstatin, 500 μM pefablock, and 1% octyl glucoside (Sigma). Following sedimentation at 14,000 × *g* (10 min at 4 °C), the supernatants were diluted 6.7-fold with a phosphate-buffered saline solution (40 mM KCl, 1.5 mM KH_2PO_4, 140 mM NaCl, 1.0 mM Na_2HPO_4, pH 7.2) and assayed by sandwich immunoassay (11) using the indicated antibodies and pure recombinant hCG as a standard. The hCG β-subunit used as a standard was purified from this hCG by high performance liquid chromatography on a C-18 resin using an acetonitrile gradient in water containing 0.1% trifluoroacetic acid as described (6). Standards were dissolved in octyl glucoside extracts of untransfected COS-7 cells for measurements of intracellular β-subunits and heterodimers. This minimized the possible influence of detergent and cell extract on the assay. Procedures to monitor assembly *in vitro* have been described (6). All sandwich assay estimates were determined statistically using Prism (GraphPad Software, San Diego, CA). Most analogs were studied three or more times. Differences in expression relative to that of hCG, which was always included as a standard, are typical despite the fact that some transfections were more efficient and led to the formation of larger amounts of heterodimers than others.

The Journal of Biological Chemistry
ASBMB
jbc
Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

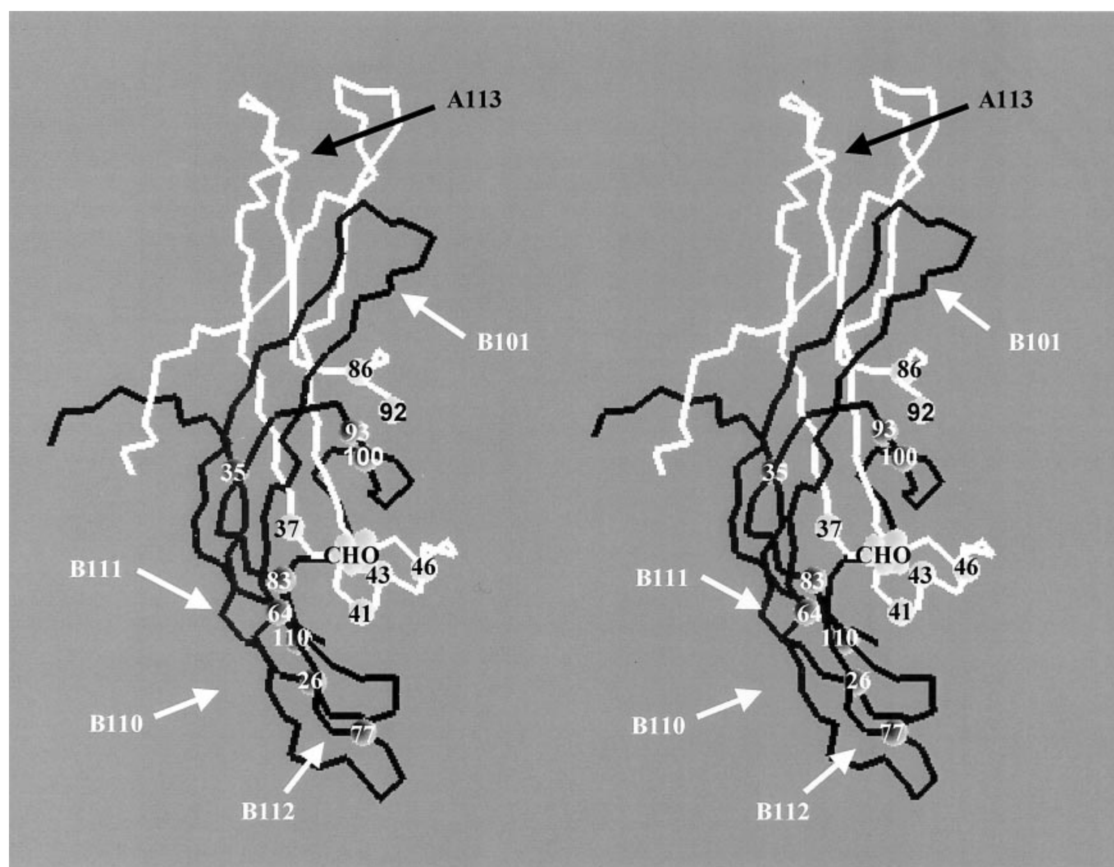

FIG. 3. **Relaxed stereo view depicting the Cα carbon atoms of hCG, the locations of mutations used in this study, and the relative antibody binding sites.** Key: α-subunit, *white*; α-subunit mutations, *light spheres with black text* denoting the amino acid residue number; *CHO*, location of residue αAsn[52] that contains an *N*-linked oligosaccharide (not shown); β-subunit, *black*; β-subunit mutations, *dark spheres with white text* denoting the amino acid residue number; *A113* and *arrow*, approximate location of the binding site of the α-subunit antibody used for capture in sandwich immunoassays; *B101*, *B110*, *B111*, *B112*, and *arrows*, approximate locations of the binding sites of the β-subunit antibodies used for capture or detection in these studies. The seatbelt latch disulfide is normally formed between β-subunit residues 26 and 110. Note, the proximity of α-subunit residues 37, 41, and 43 to β-subunit residue 26, the normal seatbelt latch site. The small seatbelt loop is stabilized by a disulfide between β-subunit residues 93 and 100. B111, an antibody that was essential to distinguish the location of the seatbelt latch disulfide, recognizes a region of hCG near β-subunit cysteines 26 and 110, although it does not recognize the seatbelt latch disulfide *per se*. Although B111 can bind to single chain analogs of hCG in which β-subunit cysteines 26 and 110 are converted to alanine, it does not recognize any analog in which the seatbelt is latched to the α-subunit (8). B111 can also recognize heterodimers in which β-subunit cysteines 26 and 110 are converted to alanine if the heterodimer is stabilized by the presence of an NH_2-terminal Fos/Jun dimerization domain or if the heterodimer is stabilized by an N-terminal disulfide cross-link (27).

RESULTS

Rationale for Our Approach to Distinguish Heterodimer Assembly Pathways—The relative timing of subunit docking and seatbelt latch formation are reversed in the threading and wraparound pathways. Therefore, in principle, the threading and wraparound pathways could be distinguished by pulse-chase analysis (7, 14, 15), the most common method for studying protein folding in mammalian cells. This approach would not be useful for detecting trace quantities of transient unstable folding intermediates such as heterodimers that have not latched their seatbelts (16) or those in which loop α2 is only partially threaded through the β-subunit hole, however. Furthermore, pulse-chase methods can give undue weight to dead-end folding products that appear transient because they are degraded, not because they are folding intermediates. These considerations led us to monitor assembly using methods that depend on the abilities of folding intermediates to compete for the formation of cross-linked heterodimers.

We distinguished the threading and wraparound pathways by measuring the amounts of cross-linked heterodimer formed when α-subunit analogs containing an additional cysteine were co-transfected with the native β-subunit (Fig. 4, *middle* and *bottom lines*). We have found that the hCG seatbelt can be latched to a cysteine added to the α-subunit during the wraparound pathway of heterodimer assembly (8). Assembly by this route is efficient and many of the cross-linked heterodimers produced are as active as hCG in receptor binding and signal transduction assays (8). During assembly that occurs by a threading pathway (Fig. 4, *lower row*), the seatbelt is latched to the cysteine in loop β1 before assembly begins. As a result, the seatbelt would remain latched to loop β1, which would lead to the formation of a heterodimer that would be unstable at pH 2, 37 °C. During assembly that occurs by a wraparound mechanism, the seatbelt would have the opportunity to be latched to the cysteine in loop β1 or that had been added to the α-subunit. Consequently, the heterodimer would contain an intersubunit cross-link.

An alternative method of distinguishing the threading and wraparound mechanisms depends on the competition between two β-subunits (Fig. 4, *top* and *bottom lines*). We employed this method to eliminate the possibility that the seatbelt latch site might be rearranged during assembly in the cell. For example, it might be possible for the seatbelt of the native hCG β-subunit to be latched to a cysteine in the α-subunit during assembly that occurs by a wraparound mechanism. Subsequently, the seatbelt latch site might "migrate" to its native latch site by a disulfide exchange with βCys[26] (Fig. 4, *dashed arrow*), making it appear that the heterodimer had been formed by a threading pathway. To avoid this possibility, we took advantage of the

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

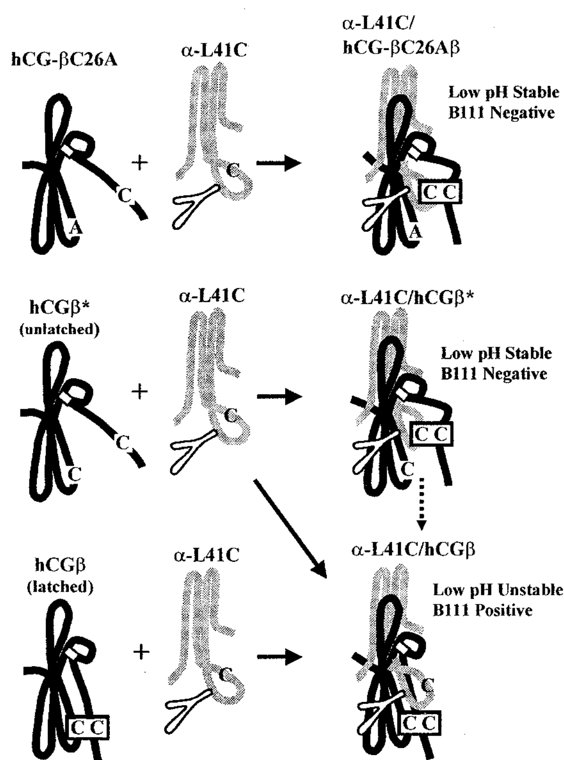

FIG. 4. **Rationale for the experiments involving a competition between hCGβ and hCGβ-C26A for α-subunit analogs that contain an additional cysteine residue.** We presume that hCGβ can exist in two states, one in which the seatbelt is latched (hCGβ) and another in which the seatbelt is unlatched (hCGβ*). Because hCGβ-C26A cannot latch its seatbelt to β1, it can exist only in a state that is comparable with hCGβ*. As outlined in the top reaction, hCGβ-C26A can form an intersubunit disulfide cross-linked heterodimer by the wraparound pathway with several α-subunit analogs that contain an additional cysteine (8). This heterodimer is stable at low pH and is readily recognized by conformation-dependent monoclonal antibodies A113, B101, B110, B112, and B122. The heterodimer is not recognized by B111, however. This shows that both subunits have folded properly but that the seatbelt is not latched as it is in hCG. hCGβ* would also be expected to form a heterodimer by a wraparound pathway. Heterodimers in which the seatbelt is latched to βCys^{26} and a cysteine added to the α-subunit would be readily distinguished by differences in their acid stabilities and their recognition by B111 as indicated. We expect that hCGβ-C26A and hCGβ* would compete for docking with the α-subunit analogs used in these studies. Because the seatbelt of hCGβ-C26A can be latched efficiently to several sites on the α-subunit (8), some seatbelts of hCGβ* should also become latched to the α-subunit. Theoretically, heterodimers in which hCGβ* is latched to a cysteine in the α-subunit could also undergo an internal disulfide rearrangement such that the seatbelt migrated from the cysteine in the α-subunit to βCys^{26}. This is indicated by a *dashed arrow*. A rearrangement such as this cannot occur for heterodimers that contain hCGβ-C26A. In the fully folded form of hCGβ, the seatbelt is latched to loop β1 and we anticipate that it would be assembled into a heterodimer only by the threading route. As shown in the text and Table I, we observed that the hCGβ-C26A seatbelt becomes latched to the α-subunit. We did not detect latching of the hCGβ seatbelt to the α-subunit or more than marginal competition of hCGβ-C26A with hCGβ for any α-subunit analog. These findings support the notions that most hCG is assembled by a threading pathway and little, if any, is formed by the wraparound pathway.

observation that the seatbelts in hCG β-subunit analogs such as hCGβ-C26A, which cannot latch their seatbelts to loop β1 because they contain an alanine in place of βCys^{26}, can become latched to cysteines added to the α-subunit in place of residues 35, 37, 41–50, 64, 86, 88, and 90–92, among others (8). Because the seatbelts of these analogs cannot be latched until after the subunits contact one another, heterodimers containing these β-subunits can only be assembled by the wraparound pathway. Furthermore, once latched, the seatbelt in these heterodimers cannot migrate to the β-subunit unless the heterodimer dissociates. The observation that these heterodimers are secreted efficiently indicates that they do not dissociate.

Before its seatbelt is latched, hCGβ would be expected to have the same overall conformation as hCGβ-C26A (Fig. 4). Thus, hCGβ-C26A and the unlatched form of hCGβ (*i.e. hCGβ**) would be expected to compete with one another for docking with the α-subunit in the wraparound pathway. If most heterodimers became assembled by the wraparound pathway, one would expect to find a significant fraction of the total heterodimer that contained hCGβ-C26A. In contrast, because hCGβ-C26A can be incorporated into heterodimers only by the wraparound pathway, it would not be expected to compete with hCGβ for heterodimers that are assembled after the seatbelt is latched, *i.e.* by threading (Fig. 4). Therefore, if most assembly occurs by threading, very little of the heterodimer formed would contain hCGβ-C26A.

The position of the seatbelt latch site in hCG can be determined by the acid stability of the heterodimer and by its ability to be recognized by monoclonal antibody B111. Heterodimers in which the seatbelt is latched to the α-subunit contain an intersubunit disulfide cross-link. These are distinguished readily from heterodimers in which the seatbelt is latched to βCys^{26} by their resistance to dissociation at pH 2, 37 °C, and by their inabilities to bind monoclonal antibody B111 (8). B111 binds a conformational hCG epitope formed when the seatbelt is latched normally, *i.e.* to βCys^{26}. It does not bind heterodimers in which the seatbelt is latched to other cysteines such as those that have been added to either subunit (8, 17).

Human Choriogonadotropin Can Be Assembled in the ER by Two Different Routes, but Most Is Made by the Threading Pathway—The seatbelts of heterodimers produced by co-expressing hCGβ and each of the α-subunit analogs tested became latched primarily to βCys^{26}, not to the cysteine added to the α-subunit. This was the first indication that hCG is formed in the ER by a threading pathway. An example of this is seen by comparing the properties of heterodimers produced when hCGβ, was expressed with α-L41C (Table I, 1). Most heterodimers secreted by cells that were co-transfected with α-L41C and hCGβ lacked an intersubunit disulfide and were unstable following 30 min at pH 2, 37 °C (Table I, 1, row 1). As a result they were detected readily in heterodimer-specific sandwich immunoassays employing an antibody to the α-subunit for capture (*i.e.* A113) and a radioiodinated antibody to the β-subunit for detection (*i.e.* ^{125}I-B110) before, but not after low pH treatment. These heterodimers were also detected in similar immunoassays employing A113 for capture and ^{125}I-B111 for detection before treatment at low pH. This indicated that their seatbelts were latched to βCys^{26}, not αCys^{41}. Both findings are consistent with the conclusion that the heterodimer was formed by a threading mechanism.

To exclude the possibility that the location of the seatbelt in the heterodimer had undergone a disulfide exchange and become latched to βCys^{26} in loop β1 after it had been latched to αCys^{41}, we repeated the study in the presence of hCGβ-C26A, an analog that cannot latch its seatbelt to loop β1. When α-L41C was expressed with hCGβ-C26A, the heterodimer that formed was detected in A113/^{125}I-B110 assays before and after acid treatment (Table I, 1, row 2). Furthermore, none of it was detected in A113/^{125}I-B111 assays before or after low pH treatment. These findings showed that seatbelt residue βCys^{110} of this heterodimer was cross-linked to the α-subunit, a consequence of its formation by a wraparound mechanism. When both β-subunits were expressed simultaneously with α-L41C, heterodimers secreted into the medium contained hCGβ and little or no hCGβ-C26A (Table I, 1, row 3). Because the seat-

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006
The Journal of Biological Chemistry

TABLE I

hCG β-subunit analogs forced to latch their seatbelts to the α-subunit compete poorly with those that can latch their seatbelts to βCys[26]

This table describes the properties of heterodimers produced by co-expressing the α-subunit analog indicated at the top of each block with β-subunits capable of participating in wrapping or threading pathways (*i.e.* hCGβ and hCGβ-KDEL), β-subunits that are limited to wrapping pathways (*i.e.* hCGβ-C26A and hCGβ-C26A-KDEL), or with both types of β-subunits simultaneously. The total amount of heterodimer in 50-μl aliquots of unconcentrated culture media (secreted) or 7 μl of cell lysate (intracellular) was quantified in an A113 capture/^{125}I-B110 detection sandwich assay using purified recombinant hCG as standard. The fraction of the total heterodimer that was acid stable was determined after treatment at pH 2 for 30 min at 37 °C. Data in the first and second columns were determined in A113 capture/^{125}I-B110 detection sandwich assays. Those in the third and fourth data columns are the results of A113 capture/^{125}I-B111 detection sandwich assays. Values in the first to fourth data columns are mean ± S.E. for 3 independent transfection plates. Similar results were observed in at least three other independent studies for each analog. The values in the fifth column, row 1 of blocks 4 and 5 were derived from these means by comparing differences in the B111 assays before and after treatment at acid pH (row 1) (Co-expression of α -subunit analogs having a free cysteine and the native hCG β -subunit can give rise to four populations of heterodimers, *i.e.* one that does not have a cross-link, one in which tensor cysteine 93 is cross-linked to the cysteine added to the α -subunit, one in which tensor cysteine 100 is cross-linked to the cysteine added to the α -subunit, and one in which the seatbelt is latched to the cysteine added to the α -subunit. We calculated the maximum amount of heterodimer in which the seatbelt is latched to the α -subunit in Table I, rows 1, 4 and 5, as follows. For simplicity, we assign variable x to be the amount of uncross-linked heterodimer, variable y to be the amount of heterodimer in which either tensor cysteine is cross-linked to the α -subunit, and variable z to be the amount of heterodimer in which the seatbelt is latched to the α -subunit. The latter could be formed only by the wraparound pathway. Constants a, b, and c represent the abilities of B111 to bind heterodimers with these configurations relative to B110, respectively. Because B111 does not bind heterodimers in which the seatbelt is latched to the α -subunit, $c = 0$. Data in the first column (C_1) is the total amount of heterodimer detected by antibody B110, namely $C_1 = x + y + z$. Data in the second column (C_2) is the fraction of heterodimer detected by antibody B110 that contains a cross-link. Thus, $C_1C_2 = y + z$. Data in the third (C_3) and and fourth (C_4) columns represent the abilities of B111 to bind the total and cross-linked heterodimers relative to those measured by B110. Note that for all calculations the values shown in C_2, C_3, and C_4 were converted to their fractional equivalents by dividing them by 100. The values of C_3 and C_4 can be expressed as: $C_3 = (ax + by + cz)/(x + y + z) = (ax + by)/(x + y + z) = (ax + by)/C_1$ and $C_4 = (by + cz)/(y + z) = by/(y + z) = by/(C_1C_2)$. To estimate maximal amount of z, we first divided C_3 by C_4 and rearranged the terms to give: $C_3/C_4 = C_2(ax + by)/(by)$. Because the ability of B111 to recognize the cross-linked heterodimer in 4 and 5 in Table I appears to be less than its ability to recognize the total heterodimer, $b \leq a$ and this equation can be written as the inequality: $C_3/C_4 \geq C_2b(x + y)/(by) = C_2(x + y)/y$, and solved for z by replacing $(x + y)$ with $(C_1 - z)$ and by replacing y with $(C_1C_2 - z)$. These relationships are known from the definitions of C_1 and C_2 noted above. Thus, $C_3/C_4 \geq C_2(C_1 - z)/(C_1C_2 - z)$, which can be rearranged to solve for z as: $z/C_1 \leq C_2(C_3 - C_4)/(C_3 - C_2C_4)$. The value of z/C_1 represents the maximal fraction of the total heterodimer that has its seatbelt latched to the α -subunit. The values of z/C_1 shown in the fifth column, row 1, of 4 and 5, were converted to percentages by multiplying them by 100). Values in the fifth column, row 2 of each data block were determined from the stability of the heterodimer. Values in the fifth column, row 3 of each data block were determined by comparing the amount of acid stable heterodimer in the absence and presence of hCGβ -C26A or hCGβ -C26A-KDEL. For example, in the case of block 2, row 3, the fraction of the acid stable heterodimer likely to contain hCGβ -C26A-KDEL was calculated as (55.78–35.50)/55.78 or 0.364. This was then used to calculate the amount of this material in the total heterodimer by multiplying it by the fraction of the total that is acid stable (*i.e.* 0.364 × 18.60%) to give the value of 6.8% shown in the table. The binding site of antibody B111 is shown in Fig. 3 and described in the corresponding legend.

β-Subunit analog	Heterodimer total/50 μl	Low pH stable heterodimer	B111/B110 Ratio total dimer	B111/B110 ratio stable dimer	Wraparound pathway, αCys to βCys[110]
	ng ± S.E.	*% Total ± S.E.*		*% Stable ± S.E.*	
1) Heterodimers containing α-L41C measured in media					
hCGβ	2.42 ± 0.23	Undetectable	65.5 ± 3.4	Not done	Undetectable
hCGβ-C26A	2.73 ± 0.23	96.5 ± 0.8	0 ± 0.3	Not done	≥96%
Both	2.13 ± 0.12	Undetectable	67.0 ± 4.3	Not done	Undetectable
2) Heterodimers containing α-L41C measured in cell lysates					
hCGβ-KDEL	6.16 ± 0.40	9.69 ± 0.39	54.66 ± 8.40	55.78 ± 3.66	Undetectable
hCGβ-C26A-KDEL	9.13 ± 0.63	103.01 ± 1.83	0.64 ± 0.18	0.50 ± 0.10	100%
Both	6.68 ± 0.57	18.60 ± 0.42	56.65 ± 8.99	35.50 ± 1.63	6.8%
3) Heterodimers containing α-L41C,N52D measured in cell lysates					
hCGβ-KDEL	3.16 ± 0.19	6.08 ± 0.45	80.64 ± 3.80	79.64 ± 4.68	Undetectable
hCGβ-C26A-KDEL	2.25 ± 0.10	110.76 ± 2.69	1.68 ± 0.57	1.78 ± 0.31	100%
Both	2.05 ± 0.08	17.30 ± 1.72	68.90 ± 10.45	43.78 ± 4.25	7.8%
4) Heterodimers containing α-S43C measured in cell lysates					
hCGβ-KDEL	5.27 ± 0.35	18.1 ± 1.0	64.10 ± 4.03	59.6 ± 1.2	≤1.5%
hCGβ-C26A-KDEL	10.92 ± 1.73	99.8 ± 18.8	0.37 ± 0.07	0.4 ± 0.1	100%
Both	6.35 ± 0.41	23.9 ± 2.0	42.47 ± 5.96	40.6 ± 0.6	7.6%
5) Heterodimers containing α-T46C measured in cell lysates					
hCGβ-KDEL	7.71 ± 0.44	14.4 ± 1.1	63.33 ± 7.04	49 ± 3	≤3.7%
hCGβ-C26A-KDEL	14.1 ± 0.65	92.8 ± 4	0.67 ± 0.03	0.8 ± 0	≥93%
Both	7.1 ± 0.55	37.5 ± 2.5	50.57 ± 3.68	21.2 ± 2.2	21.3%
6) Heterodimers containing α-S92C measured in cell lysates					
hCGβ-KDEL	9.45 ± 0.89	6.03 ± 0.4	47.77 ± 7.19	85.03 ± 4.98	Undetectable
hCGβ-C26A-KDEL	11.66 ± 1.05	128.47 ± 16.8	0.97 ± 0.12	0.37 ± 0.07	100%
Both	7.4 ± 0.31	6.60 ± 0.7	49.00 ± 1.42	65.00 ± 5.68	1.6%

belts in these heterodimers were latched to βCys[26], not αCys[41], they were detected readily in A113/^{125}I-B110 and A113/^{125}I-B111 assays before, but not after low pH treatment. The finding that hCGβ-C26A did not compete with hCGβ for formation of heterodimers containing α-L41C indicated that hCG assembly occurs primarily by threading.

One could argue that heterodimers having their seatbelts latched to αCys[41] were disrupted or degraded during secretion and that this prevented us from detecting them in preparations containing α-L41C and hCGβ. This appeared highly unlikely because heterodimers containing hCGβ-C26A were found in the medium when it was the only β-subunit used in the transfection. Nonetheless, we tested this possibility using analogs of hCGβ and hCGβ-C26A that contained four COOH-terminal residues (*i.e.* KDEL) known to delay the secretion of other proteins from the ER (18). To determine how this affected the secretion of hCG, we transfected COS-7 cells with the native α-subunit and hCGβ or hCGβ-KDEL and measured the ap-

The Journal of Biological Chemistry
ASBMB
jbc

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

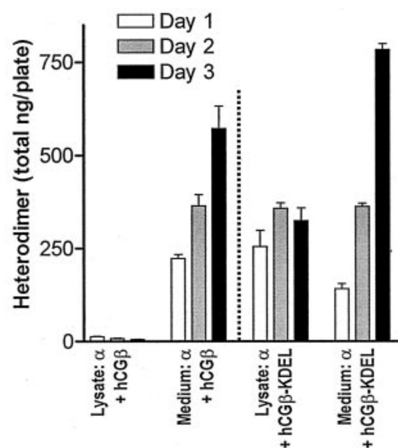

FIG. 5. **Influence of KDEL sequence on heterodimer secretion.** Cells transfected with hCG analogs containing KDEL sequences and materials that were retained within the cell (lysates) and secreted into the media during 3 days of incubation were measured as described in the text.

pearance of heterodimer in media and cell lysates. The KDEL tag delayed heterodimer secretion from the cell and caused it to be accumulated in the cells (Fig. 5). Prolonged incubation resulted in release of the KDEL-tagged material from the cells, probably because the ER retention mechanism had been saturated. These studies suggested that assays of KDEL-tagged heterodimers in cell lysates would reflect material located primarily in the ER.

The seatbelts of heterodimers measured in lysates of cells co-transfected with α-L41C and hCGβ-KDEL were latched to βCys^{26}. As a result, most were unstable at low pH and readily detected by B111 (Table I, 2, row 1). A small fraction (9.7%) of the heterodimer was stable at low pH even though it was detected in A113/^{125}I-B111 assays. Whereas its acid stability suggested that this fraction contained an intersubunit disulfide cross-link, its ability to be recognized by B111 showed that its acid stability was not due solely to the formation of a disulfide between seatbelt residue βCys^{110} and loop α2 residue αCys^{41}. This indicated that αCys^{41} can participate in an intersubunit disulfide with β-subunit cysteines other than that at the end of the seatbelt (*i.e.* βCys^{110}). The amounts of the acid stable α-L41C/hCGβ-C26A-KDEL heterodimer in cell lysates were too small for us to attempt identifying this intersubunit disulfide using traditional biochemical methods. Therefore, we modeled each of the 12 intersubunit disulfides that could be formed between αCys^{41} and cysteines in the β-subunit and excluded those that were likely to disrupt the conformation of the heterodimer to an extent that it would no longer be recognized by antibodies used in these studies. Only two intersubunit disulfides, namely αCys^{41}-βCys^{93} and, more likely, αCys^{41}-βCys^{100} appeared to fit these criteria. These β-subunit cysteines stabilize a small loop within the seatbelt and are near several residues in loop α2, including αCys^{41}.

To test the notion that βCys^{100} or βCys^{93} might participate in the formation of an intersubunit disulfide with αCys^{41} without disrupting the B111 binding site, we expressed α-L41C with hCGβ-C93A-KDEL and hCGβ-C100A-KDEL. These analogs cannot form the small seatbelt loop and have free cysteines at βCys^{100} and βCys^{93}, respectively. We also expressed α-L41C with hCGβ-C93A,C100A-KDEL, an analog lacking both cysteines. Because the small seatbelt loop has been shown to be essential for the formation of heterodimers containing the native α-subunit (16), we expected that these β-subunits would form heterodimers with α-L41C only if they could form an intersubunit disulfide. This prediction was satisfied by the observation that hCGβ-C93A-KDEL, the analog having a free cysteine at βCys^{100}, formed a heterodimer with α-L41C (Table II). The findings that α-L41C did not combine stably with hCGβ-C93A,C100A-KDEL and that the native α-subunit did not combine stably with hCGβ-C93A-KDEL supported the notion that the intersubunit disulfide involved residues αCys^{41} and βCys^{100}. As had been predicted from modeling studies, the amount of heterodimer formed when α-L41C was expressed with hCGβ-C93A-KDEL was much greater than that formed when it was expressed with hCGβ-C100A-KDEL (Table II).

Based on this observation and the finding that both the α-L41C/hCGβ-KDEL and α-L41C/hCGβ-C93A-KDEL heterodimers were detected readily by B111 (Tables I and II, respectively), we expect that the acid-stable fraction of α-L41C/hCGβ-KDEL contains an intersubunit disulfide cross-link between αCys^{41} and βCys^{100}. Nonetheless, we cannot exclude the possibility that a portion of this material contains a disulfide between αCys^{41} and βCys^{110}, indicating that it had been formed by a wraparound mechanism. The finding that the seatbelt was latched to βCys^{26} in the vast majority of the acid unstable α-L41C/hCGβ-KDEL heterodimer and a substantial fraction of the acid-stable α-L41C/hCGβ-KDEL heterodimer showed that βCys^{26} out competed αCys^{41} as a seatbelt latch site. Whereas this is consistent with the view that the seatbelt had become latched to βCys^{26} before the subunits docked, it does not preclude the possibility that the seatbelt had become latched to βCys^{26} by a process of disulfide exchange. We tested this possibility by comparing the abilities of hCGβ-KDEL and hCGβ-C26A-KDEL to compete for heterodimer formation.

The heterodimer made by co-expressing α-L41C and hCGβ-C26A-KDEL was acid stable and could not be detected in A113/^{125}I-B111 assays before or after low pH treatment (Table I, 2, row 2). This showed that it contained an intersubunit disulfide, a consequence of its assembly by a wraparound mechanism. hCGβ-C26A-KDEL barely competed with hCGβ-KDEL for heterodimer formation, however. Most heterodimers made in the presence of both β-subunit analogs were unstable at pH 2, 37 °C, and were readily detected in A113/^{125}I-B111 assays (Table I, 2, row 3). These findings are consistent with the conclusions that the seatbelts of most heterodimer molecules were latched to βCys^{26}, not αCys^{41} and that they had been formed by the threading pathway. Much of the acid-stable fraction was recognized by B111, indicating that its seatbelt was latched normally and that it may have been stabilized by an intersubunit disulfide between αCys^{41} and either βCys^{93} or βCys^{100}. Differences in the ability of B111 to recognize the acid-stable heterodimer made in transfections lacking and containing hCGβ-C26A-KDEL (*i.e.* Table I, 2, rows 1 and 3) indicated that a small amount of the acid-stable heterodimer made in the presence of hCGβ-C26A-KDEL contained an intersubunit disulfide cross-link between αCys^{41} and βCys^{110}. Based on calculations described in the legend to Table I, it appears as if 6.8% of the heterodimer in cell lysates may have been formed by the wraparound pathway when both β-subunits were present. This small amount is consistent with the notion that threading is much more efficient than wrapping, particularly because much more hCGβ-C26A-KDEL was incorporated into heterodimers containing α-L41C in the absence of hCGβ-KDEL.

We have found that removal of the loop α2 oligosaccharide facilitates threading *in vitro* (5) and considered the possibility that it would also enhance assembly in cells. To test this, we studied assembly of heterodimers that contain α-L41C,N52D, an analog of α-L41C that lacks the loop α2 glycosylation signal. We co-expressed this α-subunit analog with hCGβ-KDEL, hCGβ-C26A-KDEL, or both β-subunit analogs and measured the formation of heterodimer in cell lysates (Table I, 3). The heterodimer formed with hCGβ-KDEL was acid labile and was

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE II
Cross-linking of βCys[93] and βCys[100] to α-subunit analogs having an extra cysteine

COS-7 cells were co-transfected with constructs encoding the indicated α- and β-subunit analogs. The β-subunits employed are unable to form the tensor disulfide and in most instances did not combine stably with the α-subunit unless it contained a cysteine at an appropriate location. The free cysteine in hCGβ-C93A is βCys[100] and that in hCGβ-C100A is βCys[93]. The values under the heading B111 (% total) refer to the binding of these heterodimers by B111 relative to that of B110. These data show that βCys[100] can be cross-linked to many α-subunits containing an additional cysteine and that the resulting heterodimers can be recognized by B110 and B111. A few can also be cross-linked to βCys[93]. Not detectable refers to heterodimer that is less than 0.02 ng/50 μl. In many cases we detected the presence of material that is just above this blank value but not enough to quantify accurately. This has been identified by the symbol "<0.10."

α-Subunit	β-Subunit analog					
	hCGβ-C93A-KDEL (βCys[100])		hCGβ-C100A-KDEL (βCys[93])		hCGβ-C93A,C100A-KDEL	
	Total ng/50 μl ± S.E.	B111	Total ng/50 μl ± S.E.	B111	Total ng/50 μl ± S.E.	B111
		% total		*% total*		*% total*
Native α	Not detectable	Not detectable	Not detectable	Not detectable	Not tested	Not tested
α-L41C	0.18 ± 0.02	81.5 ± 15.3	<0.10	Not estimated	Not detectable	Not detectable
α-S43C	0.85 ± 0.03	91.2 ± 5.7	<0.10	Not estimated	<0.1	Not estimated
α-T46C	0.68 ± 0.03	62.0 ± 2.0	0.11 ± 0.01	42.9 ± 4.0	0.11 ± 0.01	98.7 ± 4.9
α-S92C	1.69 ± 0.04	107.8 ± 5.7	0.52 ± 0.07	116.7 ± 3.8	Not detectable	Not detectable

detected readily in A113/^{125}I-B111 assays, showing that its seatbelt was latched to βCys[26] (Table I, 3, row 1). Heterodimer formed with hCGβ-C26A-KDEL was acid stable and not recognized in A113/^{125}I-B111 assays, indicating that seatbelt residue βCys[110] became bridged to αCys[41], a consequence of the wraparound pathway (Table I, 3, row 2). Most heterodimers formed when both β-subunits were co-expressed with α-L41C,N52D contained hCGβ-KDEL and 80% dissociated during the pH 2 treatment (Table I, 3, row 3). The finding that most of the acid-stable heterodimer was also recognized in A113/^{125}I-B111 assays showed that its seatbelt was latched to βCys[26], not αCys[41]. Less than 8% of the heterodimer made in the presence of both β-subunit analogs appeared to contain hCGβ-C26A-KDEL. Thus, preventing the glycosylation of loop α2 did not affect the route of assembly in cells, most likely because nearly all hCG is assembled by a threading mechanism, even when the loop α2 oligosaccharide is present.

Together, these observations suggested that most heterodimers containing α-L41C are assembled by a threading route. Whereas it might be argued that the apparent inefficiency of wrapping is because of the possibility that αCys[41] is a poor seatbelt latch site, this seemed unlikely given the finding that α-L41C assembled readily with hCGβ-C26A and hCGβ-C26A-KDEL. Furthermore, we had chosen this α-subunit residue because of its proximity to βCys[26] in the heterodimer. Thus, we expected that seatbelt residue βCys[110] would be latched to αCys[41] readily, a phenomenon observed only when the seatbelt is prevented from being latched to its natural β-subunit site (*i.e.* βCys[26]).

We tested the abilities of hCGβ-KDEL and hCGβ-C26A-KDEL to compete for two other α-subunit analogs containing a cysteine in loop α2. When α-S43C and α-T46C were co-expressed with hCGβ-KDEL, we observed that 18.1 and 14.4%, of the heterodimer that remained in the cells did not dissociate at acid pH even though it was readily detected with B111 (Table I, 4 and 5, row 1). The acid stability of these heterodimers appeared because of the formation of a disulfide between the cysteine that had been added to loop α2 and βCys[93] or, more likely, βCys[100]. Both of these α-subunit analogs formed significant amounts of heterodimer when expressed with hCGβ-C93A-KDEL, indicating they are likely to contain an intersubunit disulfide cross-link with βCys[100] (Table II). Because the recognition of the acid-stable forms of these heterodimers by B111 is similar to that of heterodimers in which these α-subunit cysteines were cross-linked to βCys[100], it appeared likely that most, if not all of the cross-linked material contains a disulfide between the cysteine added to the α-subunit and either βCys[93] or βCys[100]. Consequently, the threading pathway was expected to be responsible for more than 95% of the heterodimers formed when hCGβ-KDEL was expressed with either α-S43C or α-T46C (Table I, 6 and 7, row 1).

To test the possibility that we were being mislead by our choice of an α-subunit latch site, we repeated these studies with α-S43C and α-T46C, analogs that also formed heterodimers efficiently with hCGβ-C26A (8). When these α-subunit analogs were co-expressed with hCGβ-C26A-KDEL, nearly all the heterodimer retained within the cell was acid stable (Table I, 4 and 5, row 2). Neither heterodimer was detected by B111, indicating that its seatbelt had become latched to the α-subunit. The ample amount of heterodimer formed with either α-subunit analog suggested that αCys[43] and αCys[46] are readily bridged to βCys[110]. Thus, these cysteines should have competed effectively with βCys[26] during assembly of heterodimers that formed by the wraparound pathway. The finding that little, if any, intersubunit disulfide between βCys[110] and either αCys[43] or αCys[46] was formed when α-S43C and α-T46C were expressed with hCGβ-KDEL is consistent with the notion that most heterodimers are assembled by threading (Table I, 4 and 5, row 1).

To learn if hCGβ-C26A-KDEL would compete with hCGβ-KDEL during assembly of heterodimers containing α-S43C and α-T46C, we co-expressed each α-subunit analog with both β-subunit analogs. The amounts of heterodimer formed were always similar to those seen when each α-subunit analog was co-expressed with hCGβ-KDEL alone (Table I, 4 and 5, row 3). Furthermore, we often observed that a higher percentage of heterodimers containing α-S43C and α-T46C were acid stable than when α-L41C was used as the assembly partner. For example, 23.9 and 37.5% of the heterodimer appeared to be acid stable when α-S43C and α-T46C were expressed with both β-subunit analogs (Table I, 4 and 5). B111 recognized more of the acid-stable heterodimer containing α-S43C than that containing α-T46C, an indication that the latter contained a significant fraction of hCGβ-C26A-KDEL. This difference is correlated with the amounts of heterodimer observed following transfection of the cells with hCGβ-C26A-KDEL in the absence of hCGβ-KDEL, showing that hCGβ-C26A-KDEL can compete with hCGβ-KDEL when it is present in sufficient excess. The fraction of acid-stable heterodimer in these preparations likely to contain an αCys[43]-βCys[110] or αCys[46]-βCys[110] disulfide was 7.6 and 21.3%, respectively. Note that in both cases, the presence of hCGβ-KDEL inhibited heterodimer production, indicating that heterodimer assembly occurred preferentially by a threading mechanism.

Observations made using α-subunit analogs containing a cysteine in loop α2 suggested that most hCG assembly occurred after the seatbelt had been latched. Consequently, the majority of these hCG analogs appeared to be assembled by a threading

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

route. To test the notion that cysteines in other regions of the α-subunit would permit us to detect hCG assembly by the wraparound pathway, we tested the influence of a cysteine in place of αSer[92]. We have reported that this residue can be readily latched to the seatbelt (8). This cysteine would not be expected to pass beneath the seatbelt during assembly by a threading mechanism and we reasoned that it would be less likely to form a disulfide with either βCys[93] or βCys[100] during assembly even though it can be cross-linked efficiently to either of these cysteines when the other is absent (Table II). Co-expression of α-S92C and hCGβ-KDEL led to the formation of considerable amounts of heterodimer, nearly all of which dissociated at acid pH (Table I, 6, row 1). The acid-stable fraction of this heterodimer was recognized almost as well in A113/^{125}I-B111 assays as it was in A113/^{125}I-B110 assays. Based on the abilities of α-S92C to form heterodimers with either hCGβ-C93A-KDEL and hCGβ-C100A-KDEL (Table II), we anticipate that this small amount of cross-linked heterodimer contains an intersubunit disulfide between αCys[92] and either βCys[93] or βCys[100]. Both of these are recognized well by B111. Expression of α-S92C with hCGβ-C26A-KDEL also led to the formation of a substantial amount of acid-stable heterodimer (Table I, 6, row 2), but this was not recognized by B111, indicating that it was stabilized by the αCys[92]-βCys[110] disulfide and had been formed by a wraparound pathway. Co-expression of α-S92C, hCGβ-KDEL, and hCGβ-C26A-KDEL resulted in a heterodimer that was acid unstable and recognized well by B111, indicating that it had been formed by a threading mechanism. These data suggested that only 1.6% of the heterodimer formed might contain an αCys[92]-βCys[110] disulfide. Again, this supported the notion that hCG is assembled by a threading pathway.

Human Follitropin and Thyrotropin Also Appear to be Assembled by a Threading Pathway—hFSH and hTSH are structurally similar to hCG and contain the same α-subunit. These β-subunits formed heterodimers with α-subunit analogs containing cysteines added to the α-subunit in place of residues 35, 37, 41–50, 64, 86, 88, and 90–92 (not shown). Unlike the hCG β-subunit, however, analogs of hFSH and hTSH β-subunits lacking the abilities to latch their seatbelts to β-subunit loop 1 (*i.e.* hFSHβ-C20A, hFSH-C20A-KDEL, and hTSHβ-C19A) did not form heterodimers with any of these α-subunit analogs (not shown). This suggested that the wraparound pathway makes few contributions, if any, to the assembly of hFSH or hTSH.

Latching the Seatbelt to a Cysteine in hCG β-Subunit Loop 1 Occurs Before the Subunits Dock—We began these studies to learn if threading might be capable of making small contributions to glycoprotein hormone assembly, but did not anticipate finding that it was the major route of human glycoprotein hormone assembly in the ER. Indeed, the notion that hCG is assembled by a threading mechanism contradicted earlier suggestions that it was assembled by a wraparound mechanism (7). Efforts to confirm the use of the threading pathway for hCG assembly led us to test this possibility by an alternative strategy. We reasoned that if threading is the main route of hCG assembly, the seatbelt would have a strong tendency to be latched to the β-subunit before the subunits dock, even if it were forced to be latched to sites other than βCys[26]. Furthermore, we anticipated that when the seatbelt is latched to an inappropriate site, it would interfere with heterodimer assembly. We tested these possibilities using analogs of hCGβ and hCGβ-C26A that contained an additional cysteine as described (Fig. 6).

Adding a cysteine to hCGβ creates the potential for new disulfide bond arrangements. The structure of the β-subunit suggests relatively few of these will be formed, however, without distorting the protein to the extent that it is no longer

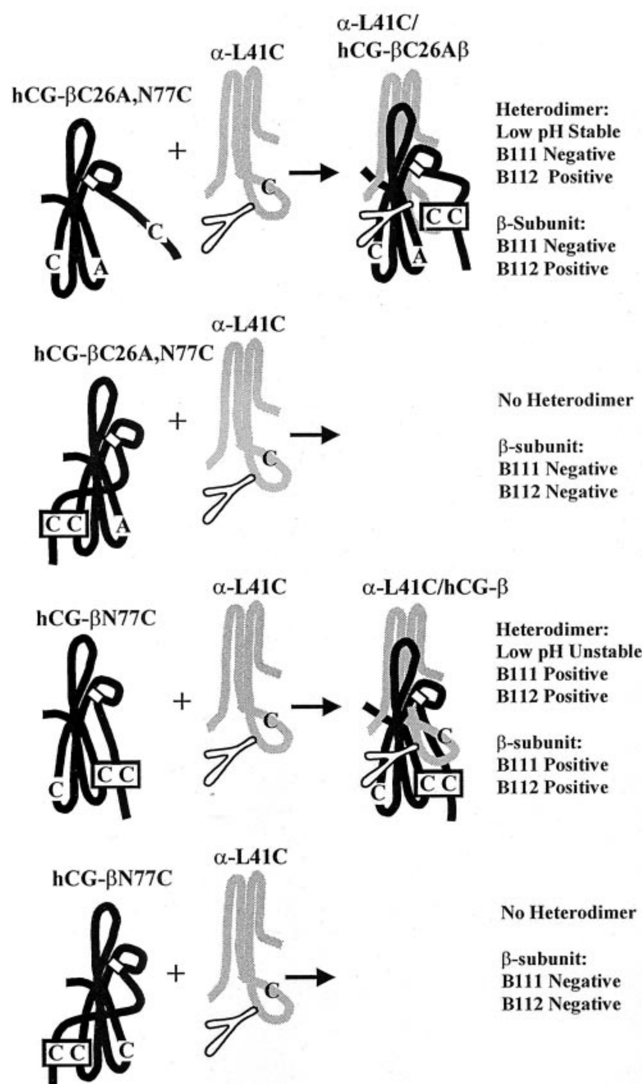

FIG. 6. **Rationale for studies involving a competition between intrasubunit cysteines and intersubunit cysteines for formation of the seatbelt latch disulfide.** The presence of an additional cysteine in hCGβ or hCGβ-C26A would create a new seatbelt latch site within the β-subunit. As shown in the *top two rows*, the presence of this cysteine in hCGβ-C26A creates an analog (hCGβ-C26A,N77C) that can exist in two states. In one of these, the seatbelt remains unlatched because of the difficulty of forming the non-native intrasubunit disulfide. In the other state, the seatbelt becomes latched to the cysteine added to the β-subunit before the subunit has time to dock with an α-subunit analog such as α-L41C. Formation of an acid-stable heterodimer would imply that the seatbelt had been latched to the cysteine added to the α-subunit. This would show that it had not become latched to the β-subunit before the subunits docked. In contrast, formation of an intrasubunit disulfide prior to docking would prevent the seatbelt from being latched to the α-subunit. Consequently, no heterodimer would form unless it were possible for the α-subunit to be threaded through the altered β-subunit. In the case of hCGβ-C26A,N77C, latching the seatbelt to βCys[77] would also disrupt the ability of the free subunit to be recognized by B112 as well as by B111. Addition of a cysteine to hCGβ to create hCGβ-N77C would provide the seatbelt with a second latch site. If the seatbelt became latched to its native site, this β-subunit would be able to form an acid unstable heterodimer by a threading mechanism and both the free subunit and the heterodimer should be detected by B111 and B112. In contrast, latching the seatbelt to the added cysteine would be expected to disrupt assembly by either the threading or the wraparound pathway. As described in the text, the presence of additional cysteines in hCGβ did not prevent heterodimer formation, suggesting that the seatbelt was more likely to form a stable disulfide with βCys[26], its natural site. In contrast, the presence of additional cysteines in hCGβ-C26A prevented this β-subunit analog from being incorporated into a heterodimer.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE III

Competition between β-subunit cysteines and a cysteine added to α2

This table describes the production of heterodimers following co-transfection of COS-7 cells in triplicate with α-L41C and the indicated β-subunit analog. The total heterodimer in the medium was determined in A113/^{125}I-B110 sandwich assays. Latching of the seatbelt to loop β1 was determined in A113/^{125}I-B111 sandwich assays. The presence of a cysteine in α-L41C reduced the binding of B111 to the α-L41C/hCGβ heterodimer to 58.9 ± 7.3% that of hCG (average of 5 independent studies). This ratio was not changed by the presence of the cysteine in the α-L41C/hCG-βN77C heterodimer. Free hCGβ-C26A,N77C was readily detected in the medium using antibody B101 for capture and ^{125}I-B122 for detection (0.67 ± 0.01 ng/50 μl medium, Study 1). hCGβ-N77C is recognized by both ^{125}I-B111 and ^{125}I-B112, antibodies that bind the hCG β-subunit near residues βCys^{110} and βAsn^{77}, respectively. This showed that the seatbelt of hCGβ-N77C is latched to βCys^{26}. In contrast, hCGβ-C26A,N77C was not recognized by either ^{125}I-B111 or ^{125}I-B112, showing that its seatbelt is latched to the cysteine introduced in place of βCys^{77}.

Analog transfected		Total dimer secreted	B111 binding	Acid stable dimer	Probable location of latch disulfide
α-Subunit	β-Subunit				
			ng/50 μl ± S.E.		
Study 1: Cysteine in place of Asn^{77} in β3					
α-L41C	hCGβ	6.52 ± 0.42	2.72 ± 0.38	<0.1	βCys^{110}/βCys^{26}
α-L41C	hCGβ-C26A	5.60 ± 0.10	<0.1	4.67 ± 0.45	βCys^{110}/αCys^{41}
α-L41C	hCGβ-N77C	2.37 ± 0.09	1.27 ± 0.09	<0.1	βCys^{110}/βCys^{26}
α-L41C	hCGβ-C26A,N77C	<0.1	Not tested	Not tested	βCys^{110}/βCys^{77}
Study 2: Cysteines in locations nearer the seatbelt origin					
α-L41C	hCGβ-C26A	1.50 ± 0.03	Not tested	1.41 ± 0.06	βCys^{110}/αCys^{41}
α-L41C	hCGβ-A35C,C26A	0.18 ± 0.01	Not tested	0.17 ± 0.01	βCys^{110}/βCys^{35}>αCys^{41}
α-L41C	hCGβ-F64C,C26A	<0.1	Not tested	<0.1	βCys^{110}/βCys^{64}
α-L41C	hCGβ-A83C,C26A	<0.1	Not tested	<0.1	βCys^{110}/βCys^{83}

measurable by conformation-sensitive antibodies such as those employed in these studies. For example, a disulfide that disrupted the cystine knot would be likely to prevent the protein from folding and being assembled into a heterodimer with the α-subunit (16). A cysteine that disrupted the disulfide between βCys^{23} and βCys^{72} would prevent it from being recognized by B110. As noted earlier, a cysteine that interfered with seatbelt latching would prevent B111 binding. Several cysteines have been added to the hCG β-subunit without preventing its assembly into heterodimers or being recognized by monoclonal antibodies (20, 21). This shows that adding a cysteine to the native hCG β-subunit does not prevent its seatbelt from being latched to βCys^{26}.

In contrast, if the seatbelt has a strong tendency to be latched to the β-subunit before the subunits dock with one another, addition of a cysteine to hCGβ-C26A would prevent it from being assembled with α-subunit analogs that contain an additional cysteine unless assembly can occur by a threading mechanism. As noted earlier, heterodimers that contain hCGβ-C26A can form only by a wraparound mechanism. The addition of a cysteine to hCGβ-C26A in place of βAsn^{77} to create hCGβ-C26A,N77C creates a potential latch site in loop β3. Because latching the seatbelt to βCys^{77} before hCGβ-C26A,N77C docks with α-L41C would prevent the seatbelt from being latched to αCys^{41}, this substitution would block heterodimer formation by a wraparound mechanism. If the latching of the seatbelt to this location also blocked threading, this substitution would also prevent any heterodimer formation (Fig. 6).

Substitution of a cysteine for βAsn^{77} in hCGβ did not prevent it from forming heterodimers with α-L41C that were recognized by B111 (Table III, Study 1). This showed that the presence of βCys^{77} did not block subunit folding or prevent the seatbelt from being latched to βCys^{26}. The total amount of heterodimer formed was usually decreased, however, indicating that βCys^{77} may have competed with βCys^{26} as a seatbelt latch site. Based on the ability of α-L41C/hCGβ-N77C to be recognized by B111, we expect that its seatbelt is latched to βCys^{26}, not βCys^{77}.

As shown earlier (Table I, 1), α-L41C can be assembled with hCGβ or hCGβ-C26A to form acid unstable and cross-linked heterodimers, respectively. We observed that addition of a cysteine to hCGβ to create hCGβ-N77C did not prevent it from being assembled into heterodimers with α-L41C (Table III, Study 1, rows 1 and 3). In marked contrast, addition of a cysteine to hCGβ-C26A, which created hCGβ-C26A,N77C, prevented heterodimer formation (Table III, Study 1, rows 2 and 4). This was not because of the inability of the cell to make this β-subunit analog. As noted in the legend to Table III, we detected a significant amount of the free β-subunit in the culture medium (0.67 ng/50 μl) that was not recognized by monoclonal antibodies B111 or B112, indicating that its seatbelt was latched to βCys^{77}. This strongly suggested that its seatbelt was latched to βCys^{77} before the subunits dock, a phenomenon that would be expected to interfere with threading. Furthermore, because βCys^{110} was present in a disulfide with βCys^{77}, the seatbelt was unable to be wrapped around loop α2 and form an intersubunit disulfide cross-link with αCys^{41}.

To minimize the possibility that there was something unique about the presence of a cysteine at β-subunit residue 77, we repeated these studies using three different analogs of hCGβ-C26A. The presence of a cysteine in place of βAla^{35}, βPhe^{64}, and βAla^{83} disrupted its ability to form cross-linked heterodimers with α-L41C (Table III, Study 2). Thus, we found 1.5 ng of heterodimer containing hCGβ-C26A in 50 μl of culture medium, but almost no heterodimer containing hCGβ-C26A analogs having cysteines in place of βAla^{35}, βPhe^{64}, or βAla^{83}. These observations supported the notion that most seatbelt latching occurred before the subunits docked. Formation of trace amounts of acid-stable heterodimer containing hCGβ-A35C,C26A (Table III, Study 2) indicated that a small fraction of the seatbelt might remain unlatched until after assembly of this heterodimer.

DISCUSSION

Threading Appears to Be the Major Route of Assembly for the Human Glycoprotein Hormones—The crystal structures of hCG (1, 2) and hFSH (3) show that their seatbelts are latched to a conserved cysteine in loop β1. Because of similarities in the locations of the cysteines of the hLH and hTSH β-subunits, the seatbelts of these hormones are also likely to be latched to this site. The efficiency with which the seatbelt can be latched to βCys^{26} of hCG suggests that the seatbelts of the other human β-subunits are also likely to be latched before their subunits dock. This is supported by our inability to detect heterodimer assembly when any of several α-subunit analogs containing an additional cysteine were expressed with hFSH and hTSH β-subunits in which βCys^{20} and βCys^{19} were replaced by ala-

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

nine. Thus, unless the seatbelt becomes unlatched transiently during the assembly process, hFSH and hTSH are also likely to be produced by a threading mechanism. Furthermore, the failure of hCGβ-C26A,N77C to be assembled into heterodimers with α-L41C suggests that the seatbelt latch disulfide is not readily disrupted once it has formed. This argues against the likelihood that the seatbelt becomes unlatched transiently during assembly.

Throughout these studies we were concerned by the possibility that we were being misled by our methodology, particularly because we had expected to find that most heterodimer assembly would occur by a wraparound pathway. We were especially cognizant of the possibility that adding a cysteine to the α-subunit might alter the route of assembly, which is why we studied several α-subunit analogs. We were also concerned that replacing hCG βCys[26] with alanine would disrupt the structure of the β-subunit. This is also unlikely based on the finding that hCGβ-C26A containing heterodimers were readily recognized by all conformation-sensitive antibodies tested except B111, an antibody that recognizes the position of the latched hCG seatbelt. Furthermore, when hCGβ-C26A or hCGβ-C26A-KDEL were expressed with cysteine containing α-subunit analogs in the absence of hCGβ or hCGβ-KDEL, they formed comparable or greater amounts of heterodimers. This indicated that hCGβ-C26A and hCGβ-C26A-KDEL were capable of being incorporated into heterodimers at rates that should have enabled them to out compete hCGβ and hCGβ-KDEL, if assembly occurs primarily by a wraparound pathway. The finding that only small amounts of hCGβ-C26A and hCGβ-C26A-KDEL were incorporated into heterodimers in the presence of hCGβ or hCGβ-KDEL suggests that the wraparound pathway is a relatively minor route for formation of the human glycoprotein hormones.

As a final effort to test the notion that threading is the more favored route of assembly, we measured the abilities of cysteines within the β-subunit to compete for formation of the seatbelt latch disulfide. The threading pathway predicts that the seatbelt would be latched prior to heterodimer assembly. The finding that addition of a latch site to hCGβ-C26A disrupted its ability to form heterodimers with α-L41C satisfied this prediction. The observation that independent methods led us to the same result, namely that threading is the dominant assembly pathway, strengthens our confidence in this conclusion significantly.

Cysteines Added to the α-Subunit Can Become Cross-linked to the β-Subunit during Assembly—A fraction of several α-subunit analogs became cross-linked to the native hCG β-subunit during assembly. This suggests that one or more β-subunit disulfides are disrupted during assembly and/or that the proximity of a β-subunit disulfide to the cysteine added to the α-subunit makes it subject to a disulfide exchange. The presence of the ER retention signal KDEL facilitated cross-linking of analogs α-L41C, α-S43C, and α-T46C (Table I, 2, 4, and 5; data for α-S43C and α-T46C expressed with β-subunits lacking KDEL retention signal not shown), indicating that this phenomenon occurred in the ER. The ability of α-S92C to be cross-linked to the native β-subunit was as low or lower than that of any other cysteine tested, even though it became cross-linked to hCGβ-C93A-KDEL and hCGβ-C100A-KDEL better than any other cysteine that was added to the α-subunit. This suggested that cross-linking depended on the proximity of the α-subunit cysteine to the seatbelt during threading. As will be described elsewhere (26), the disulfide that stabilizes a small loop within the seatbelt is disrupted during threading, which permits it to form this cross-link. This disulfide is also the only α- or β-subunit disulfide that we found to be disrupted significantly during β-mercaptoethanol catalyzed threading *in vitro* (6).

We considered the possibility that the cross-linking we observed during assembly of heterodimers that contained hCGβ and hCGβ-KDEL might be because of latching of the seatbelt to the cysteine that had been added to the α-subunit. Whereas we cannot exclude this possibility completely, the fact that the hCGβ analogs lacking the abilities to latch their seatbelts to the β-subunit competed poorly with those that can latch their seatbelts to βCys[26] in every case is inconsistent with this possibility. This includes more than 30 independent experiments done by different individuals over a period of three years, some of which were performed with 6-fold more hCGβ-C26A than hCGβ. Furthermore, the notion that the wraparound pathway is a significant mode of hCG assembly is inconsistent with the finding that the hCG seatbelt has a marked tendency to be latched to the β-subunit before the subunits dock productively, even when the seatbelt can be latched only to a site such as βCys[77] (Table III).

The Wraparound Pathway May Be a Salvage Pathway That Is Particularly Useful for Assembling Molecules with Lutropin Activity—The finding that hCGβ-C26A can be incorporated into heterodimers shows that the wraparound pathway can be used for heterodimer assembly. The observation that it is not as efficient as the threading pathway indicates that it is available as a potential salvage mechanism that would permit natural experimentation with the structure of the seatbelt. The seatbelt is the portion of the β-subunit that has the greatest influence on hormone activity (9, 22–24) and is among the most divergent parts of lutropins, follitropins, and thyrotropins (25). We were not able to detect synthesis of any hFSH or hTSH by the wraparound pathway, a phenomenon that may indicate the seatbelts of these human β-subunits are not wrapped around loop α2 efficiently. Whereas this precluded us from performing competition studies with these β-subunits, as will be discussed in greater detail elsewhere (26), cross-linked heterodimers containing either the hFSH and hTSH β-subunits were found in cells that co-express these β-subunits with α-subunits containing an additional cysteine.

Methods Used Here May Be Suited to Studying the Folding and Assembly of Other Proteins in the ER—Earlier analyses of hCG assembly *in vivo* employed pulse-chase methods that are less suited to detecting transient intermediates than the competition approach outlined here (7). This may explain why the threading pathway was not seen previously. We suggest that the competition strategies outlined here, which can be tailored to permit the detection of transient intermediates, will be useful adjuncts to pulse-chase methods for studying protein folding in cells.

Acknowledgments—We thank Dr. William Munroe for antibodies A113, B111, B112, B603, and B806, Dr. Robert Canfield for antibody B101, and Dr. Robert Campbell for purified recombinant hCG and antibody B122. We thank Dr. Robert Campbell, Dr. Ann Stock (CABM Protein Crystallography, University of Medicine and Dentistry of New Jersey), Dr. Barbara Brodsky (Biochemistry Department, University of Medicine and Dentistry of New Jersey), and Penny Swanson (Northwest Fisheries Science Center, National Oceanic and Atmospheric Administration, Seattle, WA) for reading early versions of this manuscript critically and for their valuable suggestions.

REFERENCES

1. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature* **369,** 455–461
2. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) *Structure* **2,** 545–558
3. Fox, K. M., Dias, J. A., and Van Roey, P. (2001) *Mol. Endocrinol.* **15,** 378–389
4. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50,** 465–495
5. Xing, Y., and Moyle, W. R. (2003) *Biochem. Biophys. Res. Commun.* **303,** 201–205
6. Xing, Y., Williams, C., Campbell, R. K., Cook, S., Knoppers, M., Addona, T., Altarocca, V., and Moyle, W. R. (2001) *Protein Sci.* **10,** 226–235
7. Ruddon, R. W., Sherman, S. A., and Bedows, E. (1996) *Protein Sci.* **8,**

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

1443–1452
8. Xing, Y., Lin, W., Jiang, M., Myers, R. V., Cao, D., Bernard, M. P., and Moyle, W. R. (2001) *J. Biol. Chem.* **276,** 46953–46960
9. Campbell, R. K., Dean Emig, D. M., and Moyle, W. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 760–764
10. Chen, C., and Okayama, H. (1992) *BioTechniques* **6,** 632–638
11. Moyle, W. R., Ehrlich, P. H., and Canfield, R. E. (1982) *Proc. Natl. Acad. Sci. U. S. A.* **79,** 2245–2249
12. Moyle, W. R., Pressey, A., Dean Emig, D., Anderson, D. M., Demeter, M., Lustbader, J., and Ehrlich, P. (1987) *J. Biol. Chem.* **262,** 16920–16926
13. Bernard, M. P., Cao, D., Myers, R. V., and Moyle, W. R. (2004) *Anal. Biochem.* **327,** 278–283
14. Molinari, M., and Helenius, A. (2000) *Science* **288,** 331–333
15. Lorenz, I. C., Allison, S. L., Heinz, F. X., and Helenius, A. (2002) *J. Virol.* **76,** 5480–5491
16. Suganuma, N., Matzuk, M. M., and Boime, I. (1989) *J. Biol. Chem.* **264,** 19302–19307
17. Cosowsky, L., Rao, S. N., Macdonald, G. J., Papkoff, H., Campbell, R. K., and Moyle, W. R. (1995) *J. Biol. Chem.* **270,** 20011–20019
18. Munro, S., and Pelham, H. R. (1987) *Cell* **48,** 899–907
19. Deleted in proof
20. Heikoop, J. C., van den Boogaart, P., Mulders, J. W. M., and Grootenhuis, P. D. J. (1997) *Nature Biotech.* **15,** 658–662
21. Einstein, M., Lin, W., Macdonald, G. J., and Moyle, W. R. (2001) *Exp. Biol. Med.* **226,** 581–590
22. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) *Nature* **368,** 251–255
23. Dias, J. A., Zhang, Y., and Liu, X. (1994) *J. Biol. Chem.* **269,** 25289–25294
24. Grossmann, M., Szkudlinski, M. W., Wong, R., Dias, J. A., Ji, T. H., and Weintraub, B. D. (1997) *J. Biol. Chem.* **272,** 15532–15540
25. Moyle, W. R., Myers, R. V., Wang, Y., Han, Y., Lin, W., Kelley, G. L., Ehrlich, P. H., Rao, S. N., and Bernard, M. P. (1998) *Chem. Biol.* **5,** 241–254
26. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35437–35448
27. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35458–35468

The Journal of Biological Chemistry
ASBMB
jbc ONLINE
Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 279, No. 34, Issue of August 20, pp. 35437–35448, 2004
Printed in U.S.A.
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

II. MULTIPLE ROLES OF A REDOX SENSITIVE β-SUBUNIT DISULFIDE SWITCH*

Received for publication, March 18, 2004, and in revised form, May 13, 2004
Published, JBC Papers in Press, May 25, 2004, DOI 10.1074/jbc.M403053200

Yongna Xing, Rebecca V. Myers, Donghui Cao, Win Lin, Mei Jiang, Michael P. Bernard, and William R. Moyle‡

From the Department of Obstetrics and Gynecology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson (Rutgers) Medical School, Piscataway, New Jersey 08854

All three human glycoprotein hormone heterodimers are assembled in the endoplasmic reticulum by threading the glycosylated end of α-subunit loop two (α2) beneath a disulfide "latched" strand of the β-subunit known as the "seatbelt." This remarkable event occurs efficiently even though the seatbelt effectively blocks the reverse process, thereby stabilizing each heterodimer. Studies described here show that assembly is facilitated by the formation, disruption, and reformation of a loop within the seatbelt that is stabilized by the most easily reduced disulfide in the free β-subunit. We refer to this disulfide as the "tensor" because it shortens the seatbelt, thereby securing the heterodimer. Formation of the tensor disulfide appears to precede and facilitate seatbelt latching in most human choriogonadotropin β-subunit molecules. Subsequent disruption of the tensor disulfide elongates the seatbelt, thereby increasing the space beneath the seatbelt and the β-subunit core. This permits the formation of hydrogen bonds between backbone atoms of the β-subunit cystine knot and the tensor loop with backbone atoms in loop α2, a process that causes the glycosylated end of loop α2 to be threaded between the seatbelt and the β-subunit core. Contacts between the tensor loop and loop α2 promote reformation of the tensor disulfide, which explains why it is more stable in the heterodimer than in the uncombined β-subunit. These findings unravel the puzzling nature of how a threading mechanism can be used in the endoplasmic reticulum to assemble glycoprotein hormones that have essential roles in vertebrate reproduction and thyroid function.

The glycoprotein hormones are heterodimers of two cystine knot proteins (1–3) in which a glycosylated loop of one subunit (loop α2)[1] is surrounded by a strand of the other "like a seatbelt" (1). This topology raises questions as to how these heterodimers might be assembled. We have found that the human glycoprotein hormone subunits combine by a process in which the glycosylated end of loop α2 is threaded beneath the seatbelt while it is latched (22). Although the hCG heterodimer can be assembled by a mechanism in which the seatbelt is wrapped around loop α2 after the subunits dock (4, 5), this appears to be a minor pathway that can be used to form some hormone analogs that are unable to latch their seatbelts to β-subunit loop 1. This "salvage" pathway may have had a role in the evolution of glycoprotein hormones in some teleost fish (23).

Purified glycoprotein hormone subunits have long been known to recombine slowly *in vitro* in oxidizing conditions (6), a phenomenon that occurs while all the disulfides in both subunits remain intact (7). This showed that assembly can occur by a mechanism in which the glycosylated end of loop α2 is threaded beneath the seatbelt. hCG assembly is accelerated substantially by protein-disulfide isomerase (8) and low concentrations of reducing agents, however (7). Furthermore, β-mercaptoethanol-catalyzed assembly is blocked by agents that react with thiols, *e.g.* iodoacetate (7), an indication that threading is limited by one or more disulfide bonds that must reform for the heterodimer to be stable after assembly is completed. Using a highly sensitive procedure capable of detecting and identifying trace amounts of free thiols, we found that only one of the 11 hCG disulfides was disrupted significantly during β-mercaptoethanol-catalyzed assembly (7). Because this disulfide stabilizes a small loop in the β-subunit seatbelt, this finding implies that reduction of this disulfide enhanced subunit combination *in vitro* by elongating the seatbelt. This would increase the size of the hole in the β-subunit, thereby facilitating the passage of the glycosylated end of loop α2. Whereas these studies showed how β-mercaptoethanol facilitated threading, because the reverse process must be inhibited to stabilize the heterodimer, it remained unclear as to why β-mercaptoethanol did not facilitate heterodimer dissociation.

Studies of hCG assembly in cells using pulse-chase methods (4) indicated that the disulfide that stabilizes the small seatbelt loop forms before the disulfide that latches the seatbelt to loop β1 (4). Based on our observations that most hCG is assembled while the seatbelt remains latched (22) and that this is impeded *in vitro* by the small loop in the seatbelt (7), we reasoned that the small seatbelt loop might break and reform during heterodimer assembly in the ER. Because of the transient nature of this process, we expected that formation, disruption, and reformation of this disulfide would not be detected using pulse-chase methods. To study this process in cells, we took advantage of an hCG antibody that can distinguish β-subunit isoforms in which the seatbelt is latched normally from those in which it is latched to alternate sites. By observing how free cysteines in the α- and β-subunits influence seatbelt latching and hormone assembly, we found that formation, disruption, and reformation of the disulfide that stabilizes the small seatbelt loop is critical for efficient heterodimer assembly in cells. Because this disulfide stabilizes the small loop that shortens the seatbelt, we refer to it as the "tensor." As shown here, the

* This work was supported by NICHD National Institute of Health Grants HD14907 and HD38547. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 732-235-4224; Fax: 732-235-4225; E-mail: moyle@umdnj.edu.

[1] The abbreviations used are: α2, α-subunit loop 2; β1, β-subunit loop 1; BME, β-mercaptoethanol; IA, iodoacetate; hCG, human choriogonadotropin; hFSH, human follitropin; hTSH, human thyrotropin.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

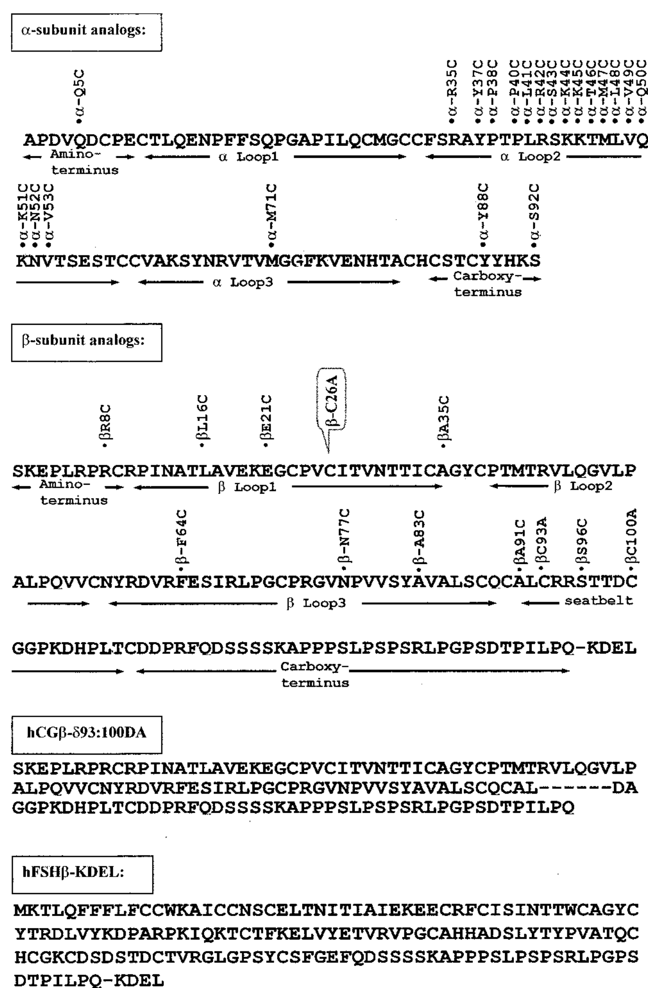

FIG. 1. **Amino acid sequences of analogs used in these studies.** The amino acid sequences of the hCG α- and β-subunits are illustrated in single letter format. The substitutions made are indicated *above* the amino acid sequence and each mutant can be identified by its name. Thus, α-Q5C represents an analog of the α-subunit in which αGln[5] was converted to cysteine. Analogs that contained the additional sequence "KDEL" were secreted slowly (22) and are presumed to be retained in the endoplasmic reticulum as has been observed for other proteins containing this signal (10). Thus, *hCGβ-C26A-KDEL* refers to an analog of the hCG β-subunit in which the natural seatbelt latch site at βCys[26] was converted to alanine and four additional residues (Lys-Asp-Glu-Leu) were fused to its COOH terminus. *hCGβ-δ93:100DA* refers to an analog of the hCG β-subunit in which residues Cys[93]-Arg[94]-Arg[95]-Ser[96]-Thr[97]-Thr[98]-Asp[99]-Cys[100], the entire tensor loop, were replaced by aspartic acid and alanine. Modeling suggested that this analog would have a seatbelt comparable in length to that of the native β-subunit when the tensor disulfide is present. The hFSH and hTSH β-subunit constructs encode the natural amino acid sequences (not shown). The hFSH-KDEL construct encodes hFSH β-subunit residues 1–108 and the sequences of hCG β-subunit residues 115–145 and KDEL fused to its carboxyl terminus to give the sequence shown.

tensor disulfide appears to function as a redox-regulated switch. Formation of the tensor disulfide facilitates latching of the end of the seatbelt to the β-subunit core. Disruption of the tensor disulfide after the seatbelt has been latched facilitates threading. Reformation of the tensor disulfide after the glycosylated end of loop α2 has been threaded beneath the seatbelt completes assembly and stabilizes the heterodimer.

EXPERIMENTAL PROCEDURES

The sources of reagents and methods of analyses used in these studies are described in the preceding manuscript (22). Constructs used in this study (Fig. 1), which were produced by standard polymerase chain reaction and cassette mutagenesis methods (9) were sequenced prior to use. The name of each construct used in these studies reflects its amino acid sequence. For example, hCGβ-C93A refers to an hCG β-subunit analog in which βCys[93] is converted to alanine. Because βCys[93] forms a disulfide in the heterodimer with βCys[100], converting βCys[93] to alanine leaves βCys[100] unpaired, making it available to form a disulfide with nearby cysteines. During these studies we prepared several hCG analogs and monitored them using well characterized monoclonal antibodies. The relative locations of the mutations and the antibody binding sites in the free β-subunit and in the heterodimer are summarized in Figs. 2 and 3. The rationale for each of the studies is described in the text and figure legends.

RESULTS

The Tensor Loop Is Usually Formed Before the Seatbelt Is Latched—Most hCG is assembled in the endoplasmic reticulum by a threading mechanism in which α2 passes beneath the seatbelt through a hole in the β-subunit (22). We had found that subunit combination was facilitated *in vitro* when the tensor disulfide (*i.e.* β93-β100) was disrupted (7) and considered the possibility that this disulfide forms in the endoplasmic reticulum only after the heterodimer is assembled. Pulse-chase analyses (4) suggested that the tensor disulfide forms before the seatbelt latch disulfide (*i.e.* β110-β26). Because the seatbelt is latched prior to hCG assembly (22), this implies that the tensor loop forms before the subunits dock, a phenomenon that would retard threading (7). Using the rationale outlined in Fig. 2, we re-examined the order in which the tensor and seatbelt latch disulfides are formed by comparing the abilities of β-subunit analogs lacking one or both tensor cysteines to latch their seatbelts to βCys[26]. Initial studies were designed to learn if the seatbelt would become latched in hCG β-subunit analogs that lacked the ability to form the tensor loop (Fig. 2, *upper panel*). To be certain that passage of the β-subunit through the secretory pathway did not influence our results, we conducted these studies using hCG β-subunit analogs that are secreted as well as those that are retained within the cell because of the presence of an ER retention signal at their carboxyl terminus (*i.e.* KDEL) (10). We have found that this signal delays heterodimer secretion (22), presumably by keeping the β-subunit in the ER.

To deduce the relative timing of seatbelt latching and tensor loop formation, we studied how adding or removing β-subunit cysteines influenced seatbelt latch formation in the free β-subunit. In these studies we quantified the total amount of β-subunit with a sandwich immunoassay using monoclonal antibodies B101 for capture and ^{125}I-B110 for detection (Fig. 3). This assay detects those molecules that have formed the β-subunit core, *i.e.* the portion of the molecule created by the formation of the cystine knot. We monitored the fraction of the total heterodimer in which the seatbelt was latched to βCys[26] using a sandwich assay employing monoclonal antibodies B101 for capture and ^{125}I-B111 for detection (Fig. 3). B111 does not recognize the hCG β-subunit when the seatbelt is latched to cysteines that are added to other sites in the β-subunit. As described next, using this property of B111, we determined that formation of the tensor disulfide usually occurs prior to formation of the seatbelt latch disulfide.

hCG β-subunit analogs lacking both tensor cysteines latched their seatbelts to βCys[26], a finding that showed that the tensor disulfide does not need to form for the seatbelt to be latched properly (Table I, rows 1 and 2). For example, B111 recognized β-subunit analogs hCGβ-C93A,C100A and hCG-C93A,C100A-KDEL, which lack cysteines βCys[93] and βCys[100] (Table I, rows 1 and 2, column C93A and C100A), as well as or better than hCGβ and hCGβ-KDEL, which contain both tensor cysteines (Table I, rows 1 and 2, column C93 and C100). This observation does not mean that the seatbelt is latched before the tensor disulfide in most β-subunit molecules, however. As described next, other data suggested that the tensor disulfide usually forms first.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

The Journal of Biological Chemistry

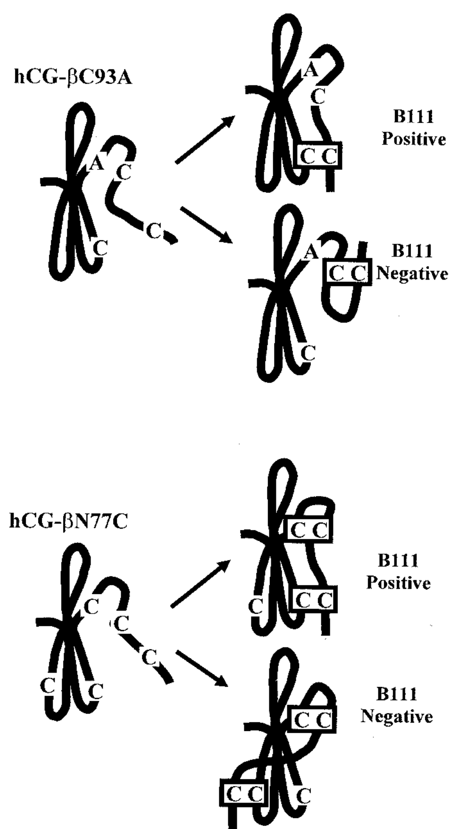

FIG. 2. **Rationale for determining the influence of the tensor disulfide on formation of the seatbelt latch disulfide.** A cysteine was introduced into the hCG β-subunit coding sequence either by changing the codons for either βCys[93] or βCys[100] to that for alanine (*top panel*) or by changing a codon for a residue in another part of the β-subunit to that for cysteine (*bottom panel*). Constructs were expressed individually in COS-7 cells and β-subunit analogs that were secreted or retained in the cells (KDEL constructs only) were measured in a sandwich immunoassay using B101 for capture and ^{125}I-B110 for detection relative to a purified hCG β-subunit standard. B110 binding provided an estimate of the total amount of β-subunit that had well folded β-core. It did not provide an indication of how the seatbelt was latched, however. Measurement of seatbelt latch formation was made using B101 for capture and ^{125}I-B111 for detection relative to the same hCG β-subunit standard. The ability of the β-subunit analogs to bind B111 relative to B110 provided an estimate of the fraction of the material in which the seatbelt is latched to βCys[26], its normal site.

To determine whether a tensor cysteine might serve as a potential seatbelt latch site, we replaced one tensor cysteine with alanine and compared the abilities of B110 and B111 to detect the resulting β-subunits. Analogs containing only a single tensor cysteine recognized B110 much better than B111, showing that the seatbelt was not latched to βCys[26] in a majority of these β-subunits. For example, only 25, 42, 14, and 24% of hCG-βC93A, hCGβ-C93A-KDEL, hCGβ-C100A, and hCGβ-C100A-KDEL subunits appeared to have their seatbelts latched to βCys[26] as reflected by differences in their abilities to be recognized by B110 and B111 (Table I, row 3–6). The loss in B111 binding relative to that of B110 caused by elimination of one tensor cysteine appeared because of the competition of the remaining tensor cysteine with βCys[26] for formation of the seatbelt latch disulfide, not to a change in folding of the remainder of the β-subunit. This is because changes in other parts of the β-subunit would have disrupted the binding of antibodies B101 and B110 to the β-subunit core. The finding that the seatbelt was not latched properly in most β-subunit molecules that contain only a single tensor cysteine (Table I, rows 3–6) is in marked contrast to the finding that the seatbelt was latched properly when both tensor cysteines were present or when both tensor cysteines were missing (Table I, rows 1 and 2). This suggests that the tensor cysteines have the potential to become latched transiently to the seatbelt before the seatbelt is latched and, as discussed next, supports the notion that the tensor disulfide is usually formed before the seatbelt is latched.

Residues βCys[93], βCys[100], and βCys[110] are located near one another in the seatbelt. Therefore, before the seatbelt is latched, βCys[110] is likely to be nearer tensor cysteines βCys[93] and βCys[100] than it is to βCys[26], the cysteine in loop β1 with which it will ultimately form the seatbelt latch disulfide. Consequently, βCys[110] may compete with βCys[93] and βCys[100] for formation of the tensor disulfide. In analogs lacking both tensor cysteines, the seatbelt has no latch site other than βCys[26],[2] which would account for the finding that it appeared to be latched exclusively to this cysteine (Table I, data rows 1 and 2). In contrast, when one of the tensor disulfides is eliminated, βCys[110] has two potential latch sites, *i.e.* βCys[26] and either βCys[93] or βCys[100]. The finding that the seatbelt was latched to βCys[26] in only a fraction of the β-subunit molecules that contained a single tensor cysteine suggests that βCys[110] has little intrinsic tendency to be latched to βCys[26]. Its proximity to βCys[93] and βCys[100] makes it more likely to form a stable disulfide with either of these tensor cysteines unless it is prevented from doing so.

What prevents the seatbelt from becoming latched stably to a tensor cysteine when both cysteines are present? The most likely explanation is that the tensor disulfide forms first or is more stable than either the βCys[110]-βCys[93] and βCys[110]-βCys[100] disulfides. Physical constraints on the positions of the latter disulfides cause them to remain near βCys[100] or βCys[93], which would enable them to be disrupted by a disulfide exchange involving βCys[100] or βCys[93], respectively. This would form the tensor disulfide and enable βCys[110] to form the seatbelt latch disulfide with βCys[26]. An exchange of this type cannot occur in β-subunit analogs that have only one tensor cysteine, which would account for the reduction in abilities of hCGβ-C100A and hCGβ-C93A to latch their seatbelts to βCys[26] (Table I, data rows 3–6). The probability that βCys[110] forms a disulfide with βCys[26] before it forms a disulfide with βCys[93] or βCys[100] can be estimated from the fraction of hCGβ-C100A and hCGβ-C93A that is recognized by B111, respectively (Table I). Whereas βCys[110] would also be capable of disrupting a disulfide between βCys[93] and βCys[100], it would be less likely to do so because its location at the end of the seatbelt does not constrain it to an area near the tensor disulfide. Thus, the findings that seatbelt latch disulfide formation is impaired by eliminating either βCys[93] or βCys[100], but not by eliminating both cysteines, suggest that the tensor disulfide forms before the seatbelt is latched in most β-subunit molecules.

The finding that removal of one tensor cysteine prevented the hCG seatbelt from being latched properly in a majority of β-subunit molecules suggested that βCys[110] at the end of the seatbelt is not constrained to a region near βCys[26]. We tested the possibility that βCys[110] scans the β-subunit to find its normal latch site by monitoring the abilities of cysteines added to the β-subunit to compete with βCys[26] for formation of the seatbelt latch disulfide. The rationale for this is described in

[2] Alternatively, one could argue that seatbelt residue βCys[110] forms a disulfide with the other cysteines in the β-subunit such as βCys[23], βCys[72], or one of those present in the cystine knot. These explanations seem unlikely, however, because the resulting analogs would not be detected using B110. The β-subunit does not form in the absence of a cystine knot cysteine (11) and is not detected by B110 when βCys[23] or βCys[72] is absent (W. R. Moyle, unpublished data). Furthermore, βCys[23] and βCys[72] are further than βCys[26] from seatbelt residue βCys[110], making them less likely to serve as seatbelt latch sites.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE I

Competition of β-subunit cysteines with βCys[26] for seatbelt residue βCys[110] and influence of the tensor disulfide

The total β-subunit was measured in sandwich assays employing antibodies B101 for capture (loop 2) and ^{125}I-B110 (loops 1 and 3) or ^{125}I-B112 (loop 3) for detection. Latching the seatbelt to βCys[26] was determined using B101 for capture and ^{125}I-B111 for detection. Purified hCGβ that had been isolated from the heterodimer was used as the standard in all assays. Values shown represent the ratio of material determined in assays employing B111 relative to that measured in assays employing B110 or B112. The distance values were measured between the Cα carbons from the start of the seatbelt βCys[90] to the free cysteine. The endoplasmic reticulum-trapped analogs contained the KDEL sequence at their carboxyl termini. Values in the column denoted "Cys[93] and Cys[100]" were for analogs that contain both tensor cysteines and that are expected to contain the tenor loop except as noted. Those in the column denoted "C93A and C100A" were for analogs in which both tensor cysteines had been replaced by alanine and that are unable to form the disulfide that stabilizes the tensor loop. All experimental values are means of triplicates ± S.E.

Data row	hCGβ analog	Distance to βCys[90]	Binding to B111 Relative to B110	
			Cys[93] and Cys[100]	C93A and C100A
		Å	*ratio ± S.E.*	
1	hCGβ	28[a]	1.16 ± 0.02	1.83 ± 0.16
2	hCGβ-KDEL	28[a]	0.86 ± 0.01	1.29 ± 0.13
3	hCGβ-C93A	8	0.25 ± 0.01	
4	hCGβ-C93A-KDEL	8	0.42 ± 0.07	
5	hCGβ-C100A	13	0.14 ± 0.02	
6	hCGβ-C100A-KDEL	13	0.24 ± 0.05	
7	hCGβ-R8C	9	1.15 ± 0.09	0.78 ± 0.15
8	hCGβ-L16C	22	0.16 ± 0.02	Not Done
9	hCGβ-E21C-KDEL	31	0.63 ± 0.10	0.42 ± 0.10
10	hCGβ-I33C	13	0.16 ± 0.01	0.05 ± 0.00
11	hCGβ-A35C	10	0.44 ± 0.03	0.14 ± 0.01
12	hCGβ-A35C-KDEL	10	0.29 ± 0.05	0.16 ± 0.01
13	hCGβ-F64C	21	0.31 ± 0.06	Not Done
14	hCGβ-F64C-KDEL	21	0.46 ± 0.01	0.51 ± 0.07
15	hCGβ-N77C	36	0.83 ± 0.14	Not Done
16	hCGβ-N77C-KDEL	36	0.47 ± 0.01	0.25 ± 0.03
17	hCGβ-A83C	18	0.37 ± 0.09	Not Done
18	hCGβ-A83C-KDEL	18	0.28 ± 0.01	0.53 ± 0.03
19	hCGβ-A91C	4	0.41 ± 0.08	Not Done
20	hCGβ-S96C	17	0.21 ± 0.02	Not Done

[a] There are no free cysteines in the hCG β-subunit and the distance illustrated is that between βCys[90] and βCys[26].

the *lower panel* of Fig. 2. Several cysteines were found to compete with βCys[26] for formation of the seatbelt latch site. These included those in place of βLeu[16], βIle[33], βAla[35], βPhe[64], βAla[83], βAla[91], and βSer[96] (Table I). For example, the presence of a cysteine in place of βLeu[16] caused a 6-fold loss in the ability of the β-subunit to be recognized by B111 relative to B110, indicating that the seatbelt was latched to βCys[26] in only one of six β-subunit molecules (*i.e.* 16% of the β-subunit). Similar results were found when the seatbelt had a choice between βCys[26] and a cysteine in place of βIle[33]. Some cysteine substitutions had relatively little influence on seatbelt latching. Thus, replacing βArg[8] or βAsn[77] with cysteine was accompanied by a smaller reduction in the abilities of the β-subunit to be recognized by B111 (Table I). With the exception of the cysteine in place of βArg[8], most cysteines competed with βCys[26] for latching the seatbelt. Consequently, addition of cysteine to most sites in loops β1 and β3 reduced the ability of the β-subunit to be recognized by B111. Cysteines that were closer to the origin of the seatbelt (*i.e.* βAla[91]) were usually more efficient competitors.[3] These observations support the notion that the hCG seatbelt does not have a strict propensity to be latched to βCys[26].

These studies supported the notion that βCys[110] "scans" the surface of the β-subunit by a constrained random walk to find βCys[26], its natural cysteine "partner." By shortening the length of the seatbelt, formation of the tensor disulfide would restrict the surface of the β-subunit that can be scanned, making it easier for βCys[110] to find βCys[26]. To test this notion, we repeated many of these studies with an hCG β-subunit analog that was unable to form the tensor disulfide. As can be seen (Table I), the ability to form the tensor disulfide influenced the abilities of cysteines to compete with βCys[26]. As a result, when the tensor disulfide was removed, there was a significant reduction in B111 binding. This indicated that the seatbelt had a greater tendency to be latched to the cysteine added to the β-subunit and a lower tendency to be latched to βCys[26]. In only one case (*i.e.* hCGβ-A83C) was the abilities of the seatbelt to be latched to βCys[26] increased when the tensor disulfide was removed. These observations are also consistent with the notion that the tensor disulfide is formed before the seatbelt is latched in most hCG β-subunit molecules.

The Tensor Loop Disulfide Appears to Be Disrupted Transiently during Assembly of Heterodimers by the Threading Pathway—Pulse-chase analyses (4) and the data just presented indicate that the tensor loop is formed before the seatbelt is latched in most hCG β-subunit molecules. Because the hCG seatbelt is usually latched prior to heterodimer assembly (22), we expect that the tensor disulfide would also be present prior to heterodimer assembly, at least transiently. Studies using purified hCG α- and β-subunits showed that disruption of the tensor disulfide facilitated assembly *in vitro* (7) and we considered the possibility that the tensor disulfide might also be disrupted during assembly in the endoplasmic reticulum. A clue to the manner in which we might identify free tensor cysteines during threading came from the observation that a fraction of some heterodimers having an additional cysteine in their α-subunits were acid stable (22). For this to occur, the cysteine in the α-subunit would need to be located near a free cysteine in the β-subunit.

As discussed elsewhere (22), two of the 12 β-subunit cysteines are more likely than the other 10 to participate in the

[3] Conceivably, residues added to the β-subunit could form an intrasubunit disulfide with one of the tensor cysteines. The resulting "loss" of a tensor cysteine would permit the seatbelt to be latched to the other tensor cysteine. While we are unable to distinguish this possibility *per se*, it seems unlikely because the tensor cysteines are much closer to one another than to any of the other cysteines in the β-subunit with the possible exception of some cysteines in the cystine knot. Furthermore, the formation of such a disulfide would not occur unless the seatbelt were sufficiently mobile that it could form a disulfide with other parts of the β-subunit. This would also suggest that the mobility of the seatbelt enables it to scan the surface of the β-subunit until it encounters a cysteine with which it can form a disulfide.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

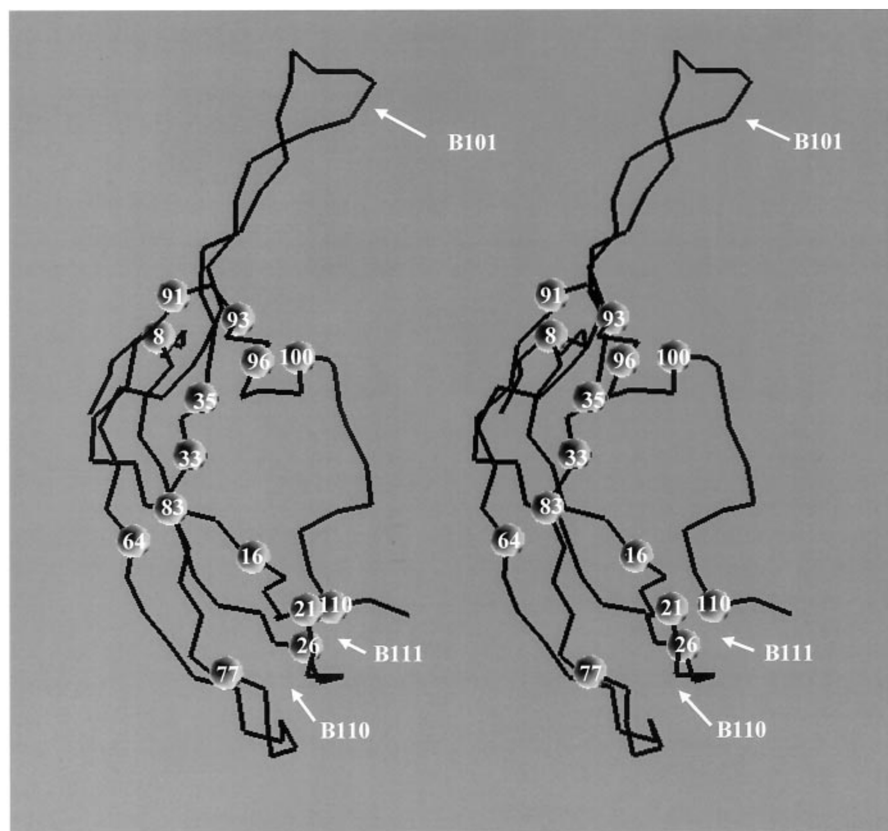

FIG. 3. **Stereo view depicting the locations of the amino acids used to create the data in Table I.** Coordinates for the Cα atoms of the β-subunit derived from the crystal structure of hCG (1) were used to make the wire diagrams shown here in *black*. The Cα atoms of the residues discussed in the text are shown *space-filled* and *numbered*. The relative binding sites of the antibodies used for measurement are also indicated. Note that the tensor disulfide is formed by residues Cys^{93}-Cys^{100} and the seatbelt latch disulfide is normally formed by residues Cys^{26}-Cys^{110}. The seatbelt extends from residues Ala^{91} to Cys^{110}. It is unlikely that the positions of β-subunit loop 2 (*top* of the figure) or the seatbelt occupy the same positions in the free β-subunit as they do in the heterodimer. Because the structure of the uncombined β-subunit is not known, this figure has been derived from the heterodimer.

intersubunit cross-link with cysteines added to the α-subunit. The finding that cross-linked heterodimers containing α-subunit analogs having an unpaired cysteine were recognized by monoclonal antibodies B110 and B111 showed that the cystine knot is intact, the seatbelt is latched properly, and the disulfide at the tips of loops β1 and β3 had not been disrupted (22). Thus, we considered it more likely that the intersubunit disulfide cross-link involved cysteines that form the tensor disulfide, the most readily reduced disulfide in the β-subunit (7), than any other β-subunit cysteines. Furthermore, modeling supported the notion that the locations of the tensor cysteines would be more likely to enable them to form disulfides with cysteines that had been added to parts of the α-subunit that passed beneath the seatbelt during threading (not shown). Our earlier studies suggested that one of the tensor cysteines can be disulfide bridged to the α-subunit (22), a finding that supports the notion that the tensor disulfide was disrupted during threading and we investigated this possibility further.

Analogs of the β-subunit that lacked one or both tensor cysteines, *i.e.* hCGβ-C93A, hCGβ-C100A, and hCGβ-C93A,C100A, were not incorporated into heterodimers containing the native α-subunit (Table II, data row 1). This confirmed earlier observations (11) and showed that the presence of a functional tensor loop was essential for heterodimer stability. In contrast, hCGβ-C93A and hCGβ-C100A, but not hCGβ-C93A,C100A, formed heterodimers with several α-subunit analogs that contained an additional cysteine (Table II, data columns 1, 2, and 4). Heterodimers that contained hCGβ-C93A are likely to contain an intersubunit disulfide between the α-subunit and βCys^{100} (Table II, data column 1). The cysteine substitution in α2 that gave rise to the most cross-linked heterodimer, *i.e.* α-S43C, is nearest βCys^{100} (1, 2). Several other cysteines that had been substituted for residues in α2 were able to participate in intersubunit cross-links with hCGβ-C93A, indicating that they can also be bridged to βCys^{100}. Cysteines that had been added to the carboxyl-terminal portions of the α-subunit also became cross-linked to this β-subunit analog, including those in place of αTyr^{88} and αSer^{92}.

Cysteines that had been added to the α-subunit were also capable of being cross-linked to βCys^{93}, but this process appeared to be less efficient than formation of a cross-link with βCys^{100}. As a result, significantly less heterodimer formed when hCGβ-C100A was co-expressed with the α-subunit analogs (Table II, data column 2). This is consistent with the crystal structure, which shows that most of the cysteines added to the α-subunit are closer to βCys^{100} than they are to βCys^{93}. It may also suggest that α2 passes nearer βCys^{100} during threading. A notable exception to this generalization was the cysteine in α-N52C. αAsn^{52} appears to be nearer βCys^{93}, a factor that may contribute to the ability of α-N52C to be cross-linked to this tensor cysteine.

We have found that the seatbelt can be latched to a cysteine added to the α-subunit (5). Whereas it might be anticipated that heterodimers containing an additional α-subunit cysteine are also stabilized by latching their seatbelts to the α-subunit, this appears unlikely. Formation of a disulfide between βCys^{110} and the cysteine added to the α-subunit was observed only when βCys^{26}, the normal seatbelt latch site, had been replaced (5). Furthermore, we found that the seatbelts of analogs containing hCGβ-C93A were latched to βCys^{26} because these acid-stable heterodimers were recognized by B111 (Table II). Presumably these analogs contain a cross-link between βCys^{100} and the α-subunit. Some of the heterodimers containing hCGβ-C100A that we presumed to have a cross-link between βCys^{93} and the α-subunit were not recognized by B111, however. Modeling suggested that it would be more difficult to form this cross-link without affecting the conformation of the heterodimer, a phenomenon that may have disrupted B111 binding. The ability of B111 to bind analogs containing hCGβ-C93A was about half of that expected relative to its ability to bind hCG. This suggested that the conformation of the seatbelt had also been altered somewhat by this cross-link.

To learn if a tensor cysteine is required for formation of a

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE II
Influence of tensor cysteines on formation of an intersubunit cross-link to a cysteine added to the α-subunit

COS-7 cells were transfected with the indicated analog and β-subunit constructs. These β-subunits are unable to form the tensor disulfide and did not combine stably with the α-subunit unless it contained a cysteine at an appropriate location. The free tensor cysteine in hCGβ-C93A is βCys[100]. That in hCGβ-C100A is β93. Some acid stable heterodimers containing hCGβ-C93A were tested for their abilities to be recognized by B111. Their recognition by B111 showed their seatbelts were latched to βCys[26], not the α-subunit. These data suggest either tensor cysteine can be cross-linked to the α-subunit, depending on the location of the added cysteine.

Data row	α-Subunit analog	β-Subunit analog			
		hCGβ-C93A	hCGβ-C100A	B111 Binding	hCGβ-C93A,C100A
		ng/50 μl ± S.E		*% hCGβ-C93A*	*ng/50 μl*
1	α	<0.10	<0.1	Not tested	Not tested
2	α-R35C	0.236 ± 0.031	<0.1	Not tested	Not tested
3	α-Y37C	0.163 ± 0.098	<0.1	Not tested	<0.1
4	α-P38C	0.333 ± 0.036	<0.1	Not tested	Not tested
5	α-P40C	0.25 ± 0.04	<0.1	Not tested	Not tested
6	α-L41C	0.28 ± 0.09	0.19 ± 0.03	Not tested	Not tested
7	α-R42C	1.18 ± 0.23	0.58 ± 0.26	Not tested	Not tested
8	α-S43C	5.26 ± 0.40	0.64 ± 0.04	41.6 ± 2.9	<0.1
9	α-K44C	0.75 ± 0.22	0.31 ± 0.15	74.7 ± 0.7	<0.1
10	α-K45C	0.70 ± 0.08	0.42 ± 0.08	55.4 ± 5.1	<0.1
11	α-T46C	2.18 ± 0.08	1.52 ± 0.05	57.7 ± 0.2	<0.1
12	α-M47C	0.35 ± 0.07	0.40 ± 0.21	46.2 ± 0.4	<0.1
13	α-L48C	3.42 ± 0.11	2.06 ± 0.04	65.2 ± 3.2	<0.1
14	α-V49C	0.60 ± 0.05	<0.1	55.2 ± 4.6	<0.1
15	α-Q50C	0.19 ± 0.02	0.37 ± 0.13	Not tested	Not tested
16	α-K51C	0.67 ± 0.12	<0.1	Not tested	<0.1
17	α-N52C	1.91 ± 0.11	1.75 ± 0.14	62.8 ± 4.9	<0.1
18	α-V53C	0.49 ± 0.05	0.18 ± 0.04	Not tested	Not tested
19	α-Y88C	8.76 ± 0.16	2.98 ± 0.22	34.5 ± 1.9	<0.1
20	α-S92C	5.55 ± 1.37	4.93 ± 0.06	Not tested	<0.1

cross-linked heterodimer, we co-expressed several α-subunit analogs with hCGβ-C93A,C100A, an hCG β-subunit analog that lacks both tensor cysteines. The trace amounts of heterodimers produced in these experiments were too small to detect.

We anticipated that formation of an intersubunit disulfide between the α-subunit and a tensor cysteine would require that cysteines added to the α-subunit be near at least one tensor cysteine. This would permit formation of a disulfide without distorting the conformation of the heterodimer so severely that it would prevent its recognition by A113 and B110. As can be seen by reference to Fig. 4, only those cysteines added to the α-subunit that are in the vicinity of the tensor cysteines were capable of forming a cross-linked heterodimer. None of the heterodimers that contained α-subunit analogs α-Q5C and α-M71C) were acid stable (Table III, data rows 2 and 14), most likely because the cysteines in these α-subunits are distant from the tensor cysteines (Fig. 4). Furthermore, being near the tensor cysteines was not sufficient to form a cross-link. Thus, whereas a cysteine at the COOH terminus of the α-subunit, αS92C, can be cross-linked efficiently to hCGβ-C93A and hCGβ-C100A (Table II, data row 20), it became cross-linked poorly to hCGβ (Table III, data row 16). This showed that formation of the cross-link occurred only while the cysteine in the α-subunit was most likely to be constrained near a free tensor cysteine or while loop α2 being threaded beneath the seatbelt.

We presumed that the disulfide formed between a tensor cysteine and a cysteine added to the α-subunit could have formed only during assembly that occurs by a threading mechanism, not during assembly that occurs by a wraparound mechanism. To test this prediction, we co-expressed α-L41C or α-S43C with the native hCG β-subunit and with hCGβ-C26A,C110A, an analog that lacks the seatbelt latch site and the cysteine at the end of the seatbelt. This analog has both tensor cysteines and would be expected to be cross-linked to either α-L41C or α-S43C if a disulfide can form between a tensor cysteine and the α-subunit while the seatbelt is unlatched. Consistent with earlier observations, co-expression of α-L41C and α-S43C with hCGβ led to a small amount of acid-stable heterodimer that was readily recognized by B111 (Table IV, rows 1 and 3). We did not detect formation of any heterodimer when α-L41C or α-S43C were co-expressed with hCGβ-C26A,C110A, however (Table IV, rows 2 and 4). Thus, formation of a cross-link between a tensor cysteine and a cysteine in loop α2 occurs only when the seatbelt is latched, most likely while the tensor disulfide is disrupted as loop α2 is being threaded beneath the seatbelt.

Only a small fraction of most heterodimers that contained an additional α-subunit cysteine was stable at acid pH (Table III). Because the remaining fraction lacked an intersubunit disulfide, it might have been formed by a threading process that occurred while the tensor disulfide remained intact. Indeed, high concentrations of the glycoprotein hormone subunits have long been known to recombine *in vitro* in oxidizing media (6), conditions in which the tensor disulfide and the seatbelt latch disulfide remain intact (7). Efforts to test the possibility that threading can occur while the tensor disulfide is intact led us to study the formation of heterodimers containing β-subunit constructs that lacked the tensor loop. These were prepared by replacing the tensor loop (*i.e.* Cys-Arg-Arg-Ser-Thr-Thr-Asp-Cys) with either Asp or with Asp and Ala. The latter substitution approximates the length of the tensor disulfide. This is seen by comparing the distance between the Cα carbons in the tensor disulfide, *i.e.* 6.2 Å, with that between the Cα atoms of a typical dipeptide, which can range from 5.3 to 6.9 Å depending on whether it is in a helix or a sheet. Molecular modeling indicated that neither of these changes would disrupt the heterodimer and that the seatbelt of the latter analog would be essentially the same length as that of the seatbelt after the tensor disulfide had formed. COS-7 cells that were co-transfected with the native α-subunit and hCGβ-δ93:100D, hCGβ-δ93:100DA, or the KDEL derivatives of these analogs failed to produce heterodimers (Table V, data for KDEL derivatives not shown). Both analogs were capable of docking with the α-subunit as shown by their ability to disrupt hCG assembly (Table V). These findings suggested that the tensor loop is essential for assembly, most likely because it enables the seatbelt to be elongated, thereby increasing the size of the hole beneath the seatbelt.

We continued to be puzzled by the observation that only a

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

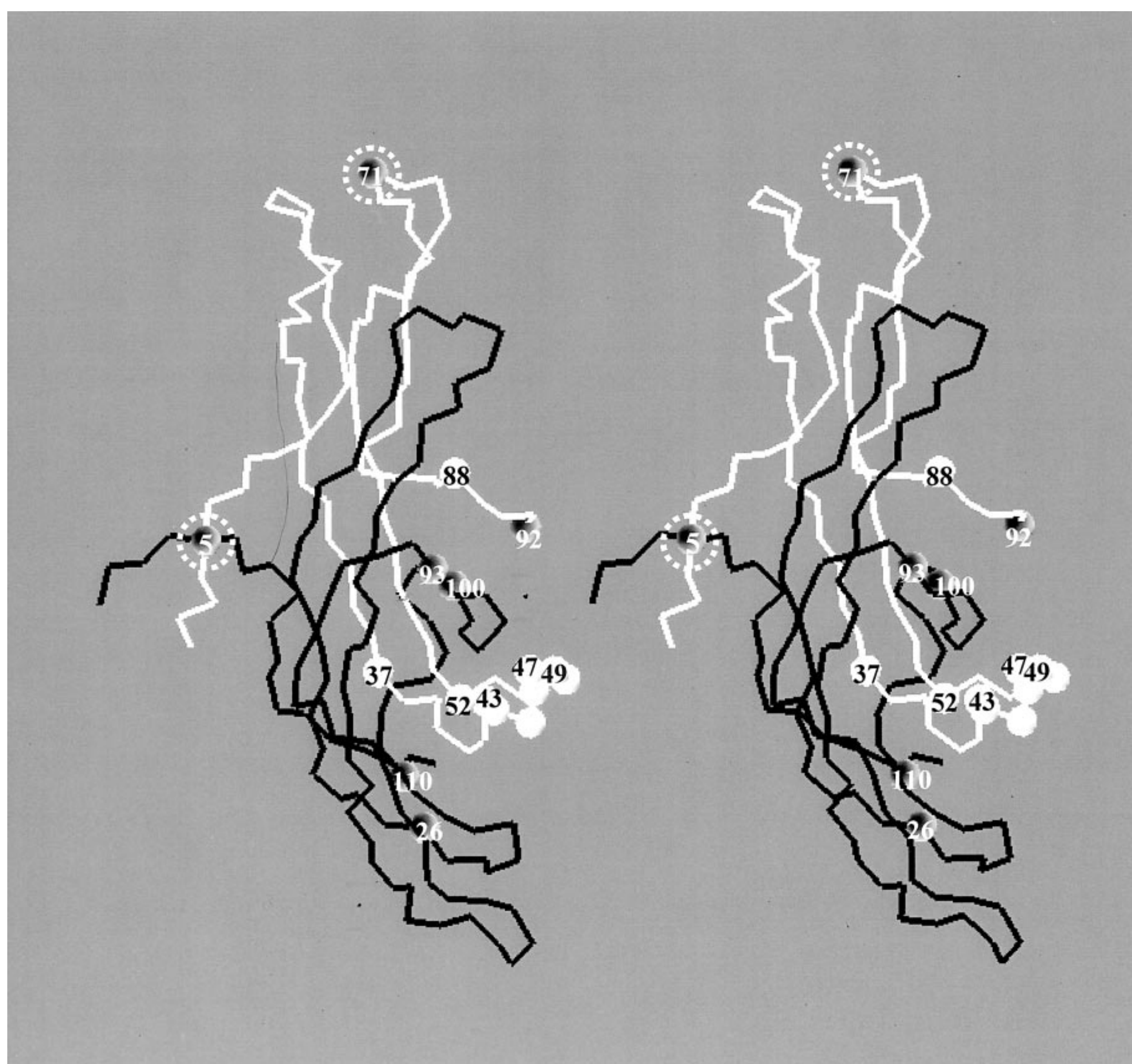

FIG. 4. **Models depicting the positions of residues that appear to form an intersubunit disulfide with an hCG β-subunit tensor cysteine relative to those that do not.** Co-expression of hCG β-subunit with some α-subunit analogs that contain an additional cysteine leads to the production of acid-stable heterodimers that are capable of binding B111 (Table III). This shows that the seatbelts of these analogs are latched to βCys[26] in loop β1, similar to that in hCG. The acid stabilities and B111 recognition properties of these heterodimers are similar to those of heterodimers containing β-subunit analogs missing one tensor cysteine and α-subunit analogs having an additional cysteine. Residues of the α-subunit that appear to form an intersubunit disulfide cross-link when expressed with the native hCG β-subunit are shown in *white with black lettering*. Not all these are labeled because of their proximity to one another. The position of the cysteine in α-S92C, which does not form an intersubunit disulfide cross-link with hCGβ but can form an intersubunit cross-link when expressed with an hCG β-subunit missing one tensor cysteine is shown in *dark gray with white lettering*. Some α-subunit analogs, such as α-Q5C and α-M71C, do not form an intersubunit disulfide with the hCG β-subunit during heterodimer assembly in the ER. These are shown in *dark gray with white lettering* and *encircled* with a *broken white line*. Note that all of these three residues do not pass under the seatbelt during threading. Residue 88 does not pass under the seatbelt either, but is constrained to be near the tensor cysteines after threading.

fraction of the heterodimers that contain an additional α-subunit cysteine were cross-linked and considered three mechanisms that would explain the formation of the intersubunit disulfide cross-link. One suggested that the disulfide formed during threading but that this was a relatively inefficient process because of the transient nature of threading. Alternatively, the intersubunit disulfide might form readily but then be disrupted as the result of a disulfide exchange caused by an attack of the other tensor cysteine that resulted in formation of the tensor disulfide. In this model, the small amount of cross-linked heterodimer that remains is a kinetically trapped reaction intermediate. The final explanation we considered was that the intersubunit disulfide formed after the heterodimer had been assembled by a disulfide exchange and was created by an attack of the α-subunit cysteine on the tensor disulfide. The first two explanations, which we could not distinguish experimentally, imply that the tensor disulfide is more stable than the intersubunit disulfide. The third explanation suggests that the intersubunit disulfide is more stable than the tensor disulfide. We reasoned that if the tensor disulfide were more stable than the intersubunit disulfide, treatment of the cross-linked heterodimers with low concentrations of reducing agent would promote a disulfide exchange leading to disruption of the intersubunit disulfide and formation of the tensor disulfide. If this occurred, the heterodimer would remain intact but no longer be acid stable. Treatment of the acid-stable fraction of several hCG analogs with 1 mM β-mercaptoethanol rendered them acid unstable without causing them to dissociate (Table VI). This showed that the intersubunit disulfide is the least stable disulfide in the heterodimer. Thus, it must have formed during assembly while the tensor disulfide had been disrupted, not by an attack of the cysteine in the α-subunit on the tensor

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006
The Journal of Biological Chemistry
ASBMB
jbc

TABLE III
Cross-linked hCG analogs detected in the ER

hCGβ-KDEL and the indicated α-subunit analogs were co-transfected into COS-7 cells and quantified in A113/^{125}I-B110 assays. Note, the analog containing M71C was quantified in A113/^{125}I-B112 assays. The fraction of cross-linked heterodimer isolated from the cells was identified by its acid stability. Heterodimer remaining after acid treatment was also detected in A113/^{125}I-B111 assays (column 3). The ability of B111 to bind this material was not quite as good as its ability to bind hCG, a phenomenon that may have reflected the influence of the cross-link on the conformation of the seatbelt. No heterodimer formed when these α-subunits were co-transfected with hCGβ-C93A,C100A-KDEL (Table II), showing that the cross-link appears to involve a tensor cysteine. The ability of B111 to bind these acid-stable analogs contrasts its inability to bind acid stable analogs in which the seatbelt is latched to the α-subunit. This showed that the acid stability of these analogs is not due to the cross-linking of βCys^{110} to the α -subunit.

Data row	α-Subunit analog	hCGβ-KDEL (both tensor cysteines present)		
		Total ER heterodimer	Acid-stable heterodimer	B111 binding to acid-stable heterodimer
		ng/10 μl ± S.E.	*% total ± S.E.*	*% stable ± S.E.*
1	α	5.46 ± 0.28	0.8 ± 0.3	Not tested
2	α-Q5C	2.58 ± 0.09	1.47 ± 0.70	Not tested
3	α-Q5C,N52D	4.07 ± 0.32	3.76 ± 0.64	Not tested
4	α-Y37C	1.21 ± 0.03	25.4 ± 2.2	Not tested
5	α-L41C	1.96 ± 0.39	8.3 ± 1.0	Not tested
6	αS-43C	6.98 ± 0.16	36.7 ± 1.8	41.6 ± 2.9
7	αK-44C	3.05 ± 0.01	11.0 ± 0.5	74.7 ± 0.7
8	αK-45C	1.35 ± 0.04	9.5 ± 0.6	55.4 ± 5.1
9	α-T46C	5.62 ± 0.27	32.7 ± 0.5	57.7 ± 0.2
10	α-M47C	5.04 ± 0.56	22.2 ± 2.1	46.2 ± 0.4
11	α-L48C	2.43 ± 0.22	22.5 ± 3.0	65.2 ± 3.2
12	α-V49C	2.36 ± 0.05	15.3 ± 0.4	55.2 ± 4.6
13	α-N52C	4.23 ± 0.50	25.3 ± 1.5	62.8 ± 4.9
14	α-M71C	4.78 ± 0.37	4.2 ± 0.8	Not tested
15	α-Y88C	7.48 ± 0.23	9.1 ± 1.3	34.5 ± 1.9
16	α-S92C	2.74 ± 0.21	1.9 ± 2.9	Not tested
17	α-M47C,N52D	4.75 ± 0.20	56.3 ± 7.7	Not tested

TABLE IV
Acid stable heterodimers containing the hCG β-subunit and α-subunit analogs having unpaired cysteines did not form unless the seatbelt was latched to βCys^{26}

Constructs encoding the indicated α- and β-subunits were transfected into COS-7 cells and heterodimer secreted into the medium was monitored by sandwich immunoassays employing α-subunit antibody A113 for detection and radioiodinated β-subunit monoclonal antibodies B110 or B111 for detection as described (22). The latter detects formation of a seatbelt latch between βCys^{26} and βCys^{110}.

α-Subunit	β-Subunit	Total dimer (B110)	pH 2 stable dimer (B110)	Total dimer (B111)	pH 2 stable dimer (B111)
		ng/50 μl ± S.E.	*% ± S.E.*	*ng/50 μl ± S.E.*	*% ± S.E.*
α-L41C	hCGβ	5.48 ± 0.51	5.19 ± 0.52	3.61 ± 0.11	3.13 ± 0.49
α-L41C	hCGβ-C26A,C110A	0.08 ± 0.01	Not tested	Not tested	Not tested
α-S43C	hCGβ	12.01 ± 0.46	11.57 ± 0.45	9.39 ± 0.15	3.75 ± 0.45
α-S43C	hCGβ-C26A,C110A	0.06 ± 0.05	Not tested	Not tested	Not tested

TABLE V
Influence of the tensor loop on heterodimer formation

COS-7 cells were transfected with constructs encoding the native human α-subunit and hCGβ or the indicated hCG β-subunit analogs. Heterodimer formation was monitored in A113/^{125}I-B110 sandwich immunoassays. Analogs hCGβ-δ(93:100)D and hCGβ-δ(93:100)DA, which lack the tensor loop were detected in B111 assays indicating that their seatbelts are latched to βCys^{26} (not shown). The seatbelt of hCGβ-C93A,C100A is also known to be latched to βCys^{26} based on its ability to be recognized by B111 (Table I).

Constructs used in transfection	Heterodimer (total/50 μl)
	ng ± S.E.
α + hCGβ	1.80 ± 0.05
α + hCGβ-C93A,C100A	Not detected
α + hCGβ + hCGβ-C93A,C100A	0.23 ± 0.01
α + hCGβ-δ(93:100)D	Not detected
α + hCGβ + hCGβ-δ (93:100)D	1.16 ± 0.29
α + hCGβ -δ(93:100)DA	Not detected
α + hCGβ + hCGβ-δ(93:100)DA	0.21 ± 0.00

disulfide after assembly had been completed.

A fraction of the heterodimer produced when hFSH and hTSH β-subunits were co-expressed with some α-subunit analogs containing an additional cysteine was also stable at acid pH (Table VII). This suggested that similar to hCG, analogs of hFSH and hTSH that have an additional cysteine in their α-subunits can become cross-linked by an intersubunit disulfide. As in the case of the hCG analogs just discussed, we observed good cross-linking with α-S43C. This is most likely because of its proximity to hFSHβ-Cys^{94} or hTSHβ-Cys^{95}, which correspond to hCGβ-Cys^{100}. Substitution of cysteines for residues αThr^{46}, αMet^{47}, αLeu^{48}, and αVal^{50} also resulted in the formation of intersubunit disulfides with all three glycoprotein hormone β-subunits. These residues are also located in loop α2 and would pass beneath the seatbelt during threading. We noted some differences in the residues that appeared to be cross-linked in hCG, hFSH, and hTSH, however. For example, we did not detect the formation of any cross-link containing α-K45C in hFSH and hTSH (Table VII, legend), an analog of the α-subunit that led to the formation of small amount of cross-linked heterodimers containing hCGβ-KDEL (Tables III and VII). We also failed to detect the formation of hFSH or hTSH analogs that contained a cross-link with α-Y88C. This may reflect subtle differences in the pathways taken by α2 during threading of each type of heterodimer. It may also reflect differences in the compositions of the β-subunits, notably their seatbelts, which is the portion of the β-subunit primarily responsible for its influence on biological activity (9, 12–14). We were unable to characterize the hFSH and hTSH analogs beyond their acid stability because of the lack of an antibody that recognized the seatbelt latch disulfide in these hormone analogs and did not study them further.

Heterodimer Assembly Appears to Be Driven by Differences in the Redox Potential of the Tensor Disulfide in the β-Subunit and the Heterodimer—Based on the known mobility of loop α2 (15), we envision that threading involves motions of this loop and

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE VI

Influence of mild reduction on cross-linked heterodimers

The acid-stable fraction of heterodimers, which were presumed to contain a disulfide cross-link between a tensor cysteine and a cysteine added to α2, were treated with 1 mM BME. The resulting material was tested for its acid stability and its ability to be recognized by B111 in A113/B111 sandwich immunoassays. These data show that mild reduction disrupted the intersubunit cross-link and rendered the heterodimer acid unstable. We suggest this is due to the disruption of the intersubunit disulfide caused by formation of the tensor disulfide.

Cross-linked heterodimer	Initial heterodimer	Amount after BME	Cross-linked after BME	Disulfide exchange
		ng/50 μl		*%*
α-S43C + hCGβ-KDEL	17.31	10.57	3.12	71
α-T46C + hCGβ-KDEL	13.50	10.53	0.83	>90
α-M47C + hCGβ-KDEL	5.03	3.60	0.20	>95
α-L48C + hCGβ-KDEL	4.01	3.05	<0.1	100

TABLE VII

Cross-linked hFSH and hTSH heterodimers

COS-7 cells were transfected with the indicated α-subunits and hFSHβ, hFSHβ-KDEL, and hTSHβ subunits. A fraction of several of these heterodimers was acid stable indicating that it was cross-linked. In addition to the analogs described here, we tested several other analogs with these β-subunit analogs, none of which contained a significant amount of cross-link. These included: α-R35C, α-R42C, α-K45C, α-Q50C, α-Y88C, α-H90C, and α-S92C.

α-Subunit analog	Stability at acid pH		
	hFSHβ	FSHβ-KDEL	TSHβ
		% ± S.E.	
α-L41C	Not detected	Not detected	19.3 ± 1.9
α-S43C	Not detected	33.5 ± 3.7	37.7 ± 1.6
α-K44C	Not detected	Not detected	24.2 ± 1.1
α-T46C	14.0 ± 1.3	Not done	25.1 ± 1.6
α-M47C	13.9 ± 1.3	27.3 ± 1.9	14.5 ± 2.2
α-L48C	Not detected	44.9 ± 0.8	14.3 ± 0.7
α-V49C	Not detected	32.9 ± 7.0	16.4 ± 1.6

parts of the seatbelt similar to those in a molten globule. Once threading is complete, reformation of the tensor disulfide would be expected to stabilize the heterodimer. Indeed, hCG β-subunit analogs that are unable to latch their tensor disulfides are not stable (11) unless they are secured by some other mechanism such as an intersubunit disulfide bond (Table II). This suggests that the tensor loop has a key role in stabilizing the heterodimer.

To learn if the tensor disulfide is more stable in the heterodimer than in the free β-subunit, we treated equimolar amounts of hCG and hCGβ with 0–2 mM BME for 15 min at 37 °C and then blocked the resulting free thiols with an excess of iodoacetate (IA). Consistent with the finding that treatment of hCGβ with low concentrations of BME disrupted only its tensor disulfide (7), BME/IA treatment blocked the ability of hCGβ to combine with the α-subunit (Fig. 5*a*), but did not alter its recognition by conformation-dependent antibodies B101, B111, or B112 (Fig. 5*b*). The abilities of these antibodies to recognize the β-subunit showed that mild reduction and alkylation did not affect the subunit core or the seatbelt latch disulfide. The finding that BME/IA treatment altered the ability of the β-subunit to be incorporated into heterodimers is consistent with the known sensitivity of the tensor disulfide to reduction (7). In contrast, similar BME/IA treatment did not influence the stability of hCG (Fig. 5*a*) or its ability to be recognized by these antibodies (Fig. 5*b*). If these concentrations of BME had disrupted the tensor disulfide in the heterodimer, they would have rendered βCys^{93} and/or βCys^{100} capable of being alkylated and thereby promoted heterodimer dissociation. The finding that the free β-subunit was rendered incapable of being incorporated into the heterodimer by concentrations of BME/IA that had no influence on the stability of the heterodimer shows that the tensor disulfide is more stable in the heterodimer than in the free β-subunit. The increased stability of the tensor disulfide to reducing agents shows how assembly of the heterodimer can be driven by the redox potential of the ER. Disruption of the tensor loop would permit threading during assembly. The increased stability of the tensor disulfide following the completion of threading and heterodimer formation (Fig. 5) would prevent loop α2 and its attached oligosaccharide from passing beneath the seatbelt, a process needed to reverse assembly.

DISCUSSION

The Tensor Disulfide Has Multiple Roles in Heterodimer Assembly, the First of Which Is to Facilitate Formation of the Seatbelt Latch Disulfide—Before it is latched, the end of the seatbelt appears to be a highly mobile portion of the hCG β-subunit. This explains the ability of hCGβ-C26A, an analog lacking the normal β1 seatbelt latch site, to latch its seatbelt to cysteines introduced into the α-subunit (5) or into other parts of the β-subunit (22). It also accounts for the abilities of cysteines that are introduced into the β-subunit to compete with βCys^{26} as a seatbelt latch site before the subunits dock (Table I). Except for cysteines added to some portions of the amino-terminal end of the β-subunit, those that are within the distance capable of being reached by βCys^{110} can serve as seatbelt latch sites. Their abilities to compete with βCys^{26} as a latch site appears to vary inversely with their distance from βCys^{90}, the residue that tethers the amino-terminal end of the seatbelt. This suggests that the seatbelt latch disulfide forms after a constrained random walk over much of the β-subunit surface, not a predetermined fold that puts it adjacent to βCys^{26}.

The findings that the seatbelt is mobile and that it can become latched to the tensor cysteines creates potential problems for latching the seatbelt to βCys^{26}. As shown (Table I), either tensor cysteine (*i.e.* βCys^{93} or βCys^{100}) can compete with βCys^{26} for latching the seatbelt. Indeed, the proximity of the tensor cysteines to βCys^{110} makes it likely that the seatbelt would become latched to either βCys^{93} or βCys^{100} before it becomes latched to βCys^{26}. Indeed, the apparent ability of hCGβ-C93A,C100A to be recognized better than hCGβ in B111 assays than in B110 assays (Table I) might reflect the possibility that the seatbelt becomes latched to either βCys^{93} or βCys^{100} in some hCGβ molecules expressed in cultured cells. Nonetheless, we cannot exclude the possibility that differences in B111 recognition occur because of differences in the conformations of the seatbelt caused by the presence and absence of the tensor loop. The problem caused by improper latching of the β-subunit would be largely avoided if the tensor disulfide forms rapidly, thereby preventing βCys^{93} and βCys^{100} from serving as potential seatbelt latch sites. Furthermore, the proximity of the tensor cysteines would be expected to facilitate a disulfide exchange that disrupts an inappropriate disulfide between the other tensor cysteine and seatbelt residue βCys^{110}. This would result in formation of the tensor disulfide before the seatbelt latch disulfide.

In addition to eliminating the potential competition between the tensor cysteines and the seatbelt, formation of the tensor disulfide would shorten the seatbelt. This would reduce the area of the β-subunit that can be scanned by the end of the seatbelt before it becomes located in the vicinity of βCys^{26} and,

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

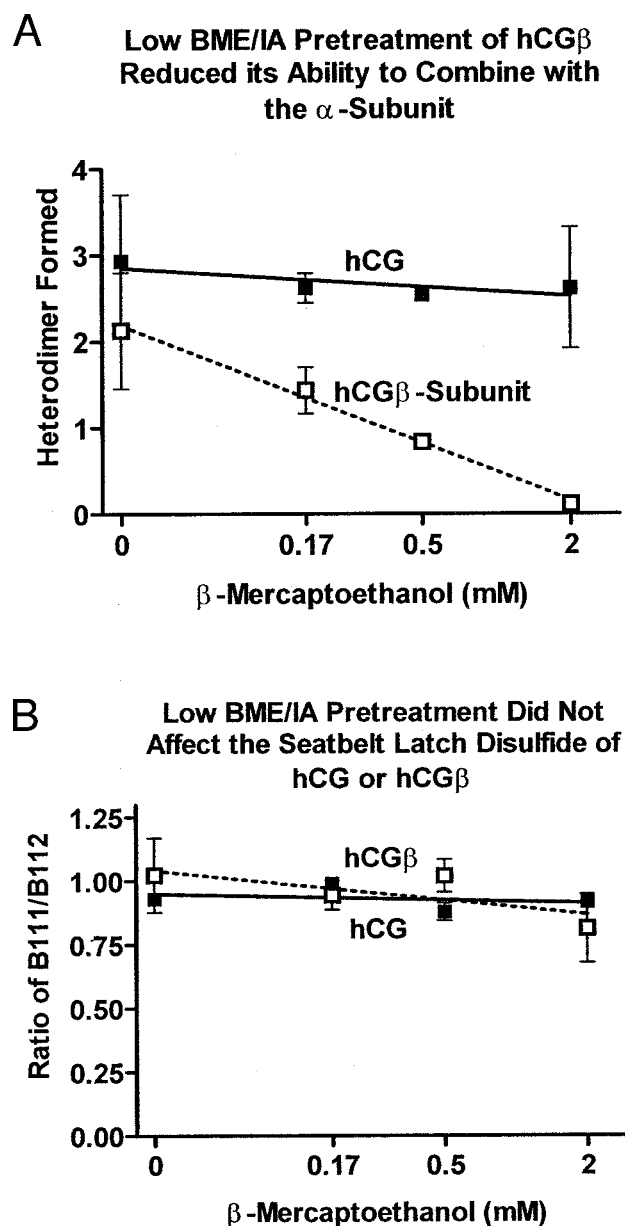

FIG. 5. **Influence of BME and IA treatment on hCG and the free β-subunit.** Equimolar amounts of hCG and free β-subunit (10^{-10} mol/5 μl, 2×10^{-7} M) were treated with 0, 0.17, 0.5, and 2.0 mM β-mercaptoethanol (15 min, 37 °C). The reaction was terminated by the addition of iodoacetate (final concentration, 10 mM) and aliquots were taken to determine the amount of hCG that had dissociated and the ability of the β-subunit to combine with the α-subunit. These low concentrations of BME did not promote subunit dissociation, measured in A113/B111, A113/B112, B101/B111, and/or B101/B112 sandwich assays (not shown). The higher concentrations of BME followed by IA treatment blocked the ability of the free β-subunit to combine with the α-subunit, $p < 0.003$ (*A*), but had no influence on the seatbelt latch disulfide of hCG or the free β-subunit detected as the ratio of B111/B112 binding (*B*). The amount of hCG recovered during the recombination study was 78% of the theoretical limit.

as a consequence, it would facilitate latch formation, as was found (Table I). Considered together these findings suggest that the tensor disulfide forms before the seatbelt is latched, at least transiently.

Disruption and Reformation of the Tensor Disulfide Have Key Roles in Heterodimer Assembly—Most human glycoprotein hormone heterodimers are assembled by a process in which a part of the α-subunit and its attached oligosaccharide are threaded beneath the seatbelt through a hole in the β-subunit (22). As shown here, assembly of hCG in the endoplasmic reticulum is assisted by disruption and reformation of the tensor disulfide. Disruption of the tensor disulfide elongates the seatbelt, which is expected to facilitate passage of loop α2 and its associated oligosaccharide beneath the seatbelt during threading. Reformation of the tensor disulfide following threading shortens the seatbelt and would be expected to retard heterodimer dissociation by hindering the glycosylated end of loop α2 from passing through the hole in the β-subunit.

The role of the tensor disulfide in assembly was first detected during studies to uncover the mechanism by which reducing agents potentiate hCG assembly *in vitro*, a process found to occur exclusively by threading (7). The tensor disulfide was reduced more readily than any other disulfide in the hCG α- or β-subunits and was essentially the only disulfide disrupted by concentrations of reducing agents that facilitated assembly optimally (7). The finding that the tensor disulfide is more stable in the heterodimer than in the free β-subunit (Fig. 5) showed that concentrations of reducing agents sufficient to disrupt the tensor disulfide before assembly is initiated would not prevent reformation of the tensor disulfide once assembly is completed. This explains the ability of a mild reducing environment to potentiate hCG assembly *in vitro* and in the endoplasmic reticulum. Thus, the tensor disulfide can be viewed as a redox-sensitive switch that opens before the subunits have combined and closes afterward to secure the heterodimer. By itself, disruption and reformation of the tensor disulfide would not be sufficient to drive assembly of the heterodimer, however. As described elsewhere (24), contacts between the amino-terminal portions of the hCG subunits and between α-subunit loops 1 and 3 with a portion of β-subunit loop 2 appear to have key roles in subunit docking. Based on the crystal structures of hCG and hFSH, which reveal several hydrogen bond contacts between the backbones of loop α2 and portions of the β-subunit cystine knot, we suggest that formation of these hydrogen bonds drives the migration of loop α2 under the seatbelt to create an unstable heterodimer (Fig. 6). Contacts between the tensor loop and loop α2 appear to stabilize the tensor disulfide, which then stabilizes the heterodimer.

We tested the notion that hCG assembly in the endoplasmic reticulum requires transient disruption of the tensor disulfide using β-subunit analogs in which the small loop in the seatbelt was replaced with either aspartic acid or aspartic acid and alanine. Molecular modeling showed that the length of the seatbelt in these analogs would be similar to that in hCG when the tensor disulfide is formed. In principle, because the hCG subunits can combine while the tensor disulfide remains intact (7), β-subunits having a dipeptide in place of the small seatbelt loop would be expected to combine with the α-subunit during assembly in cells unless this process requires disruption of the tensor disulfide. Neither of the β-subunits that contained an Asp or Asp-Ala in place of the tensor loop were incorporated into heterodimers even though each appeared to latch their seatbelts[4] and to dock with the α-subunit (Table V). This suggested that increasing the length of the seatbelt by disrupting the tensor disulfide, a phenomenon that enhanced assembly *in vitro* (7), may be crucial for heterodimer assembly in the endoplasmic reticulum.

[4] hCGβ-δ93:100DA, an hCG β-subunit analog in which the tensor loop is replaced by aspartic acid and alanine, was readily secreted from cells and detected in assays employing B101 for capture and either ^{125}I-B110 or ^{125}I-B111 for detection. This indicated that except for the absence of the tensor loop, its structure was similar to that of the hCG β-subunit and its seatbelt had been latched normally. We were unable to detect any heterodimer when either hCGβ-δ93:100DA or an analog having the KDEL endoplasmic reticulum retention signal were coexpressed with the α-subunit.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 34, Issue of August 20, pp. 35449–35457, 2004
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in U.S.A.

Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

III. THE SEATBELT AND ITS LATCH SITE DETERMINE THE ASSEMBLY PATHWAY*

Received for publication, March 18, 2004, and in revised form, May 13, 2004
Published, JBC Papers in Press, May 25, 2004, DOI 10.1074/jbc.M403054200

Yongna Xing, Rebecca V. Myers, Donghui Cao, Win Lin, Mei Jiang, Michael P. Bernard, and William R. Moyle‡

From the Department of Obstetrics and Gynecology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson (Rutgers) Medical School, Piscataway, New Jersey 08854

Vertebrate glycoprotein hormone heterodimers are stabilized by a strand of their β-subunits known as the "seatbelt" that is wrapped around loop 2 of their α-subunits (α2). The cysteine that terminates the seatbelt is "latched" by a disulfide to a cysteine in β-subunit loop 1 (β1) of all vertebrate hormones except some teleost follitropins (teFSH), wherein it is latched to a cysteine in the β-subunit NH_2 terminus. As reported here, teFSH analogs of human choriogonadotropin (hCG) are assembled by a pathway in which the subunits dock before the seatbelt is latched; assembly is completed by wrapping the seatbelt around loop α2 and latching it to the NH_2 terminus. This differs from hCG assembly, which occurs by threading the glycosylated end of loop α2 beneath the latched seatbelt through a hole in the β-subunit. The seatbelt is the part of the β-subunit that has the greatest influence on biological function. Changes in its sequence during the divergence of lutropins, follitropins, and thyrotropins and the speciation of teleost fish may have impeded heterodimer assembly by a threading mechanism, as observed when the hCG seatbelt was replaced with its salmon FSH counterpart. Whereas wrapping is less efficient than threading, it may have facilitated natural experimentation with the composition of the seatbelt during the co-evolution of glycoprotein hormones and their receptors. Migration of the seatbelt latch site to the NH_2-terminal end of the β-subunit would have facilitated teFSH assembly by a wraparound mechanism and may have contributed also to its ability to distinguish lutropin and follitropin receptors.

The glycoprotein hormones have key roles in reproduction and thyroid function (1). These heterodimers have an unusual topology in which a strand of their β-subunits surrounds a loop of their α-subunits (2–4). Because the carboxyl-terminal end of this strand is "latched" by a disulfide to the β-subunit, it has been likened to a "seatbelt" (2). In addition to its role in stabilizing the heterodimer, the seatbelt is responsible for much of the influence of the β-subunit on human glycoprotein hormone activity (5–8).

Most glycoprotein hormone β-subunits appear to have evolved from a single ancestor (9) and their folding pattern is highly conserved in all vertebrates except for that in FSH[1] of some teleost fish. Whereas in most species the seatbelt is latched to a cysteine in loop β1, in many teleosts it is latched to a cysteine in the NH_2-terminal end of the β-subunit corresponding to hCG residue βLeu^5 (10). This reduces the size of the hole in the β-subunit through which loop α2 is straddled (Fig. 1). The smaller size of this space in teFSH would be expected to impede threading of the glycosylated end of loop α2 through the β-subunit, a phenomenon that explains the greater acid stability of the teFSH heterodimer (10, 11) relative to that of glycoprotein hormones such as hCG. The latter dissociate completely within 15–20 min at pH 2, 37 °C (12).

The human glycoprotein hormones hCG, hFSH, and hTSH are assembled in mammalian cells primarily by a process in which loop α2 and its attached oligosaccharide are threaded through a hole in the β-subunit. This hole is formed after the seatbelt is latched to loop β1 (19, 20). The smaller space available for passage of loop α2 beneath the teFSH seatbelt suggested that teFSH might be assembled by a mechanism in which the subunits dock before the seatbelt is latched. This would enable the seatbelt to be wrapped around loop α2 before it is latched to a cysteine in the β-subunit NH_2 terminus. Experiments described here were designed to learn how teFSH analogs of hCG are assembled in the endoplasmic reticulum and to identify factors that contribute to the use of the threading and wraparound pathways for glycoprotein hormone assembly. Because the topology of teFSH differs substantially from that of hCG, we anticipated these studies would also enable us to test our procedures for analyzing hormone assembly in living cells (19, 20). By studying the assembly of a series of salmon FSH-hCG chimeras that are readily monitored using antibodies to hCG, we found that hCG analogs having the teFSH fold cannot be assembled by a threading mechanism and, as a consequence, are formed by a wraparound mechanism. Forcing the seatbelt to be latched to a site in the NH_2 terminus enhanced the assembly of teFSH analogs, most likely by reducing the inherent tendency of the seatbelt to be latched before the subunits dock.

EXPERIMENTAL PROCEDURES

The α- and β-subunit analogs used in these studies are illustrated in Fig. 2. Chimeras of salmon FSH and hCG β-subunits are identified using the root term s/hCGβ. For example, s/hCGβ-Nt,SB,C26A is an hCG β-subunit analog that contains the salmon FSH NH_2 terminus and seatbelt and an alanine in place of βCys^{26}. All other reagents and

* This work was supported by NICHD National Institutes of Health Grants HD14907 and HD38547. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 732-235-4224; Fax: 732-235-4225; E-mail: moyle@umdnj.edu.

[1] The abbreviations used are: FSH, follitropin; hCG, human choriogonadotropin; hFSH, human follitropin; hTSH, human thyrotropin; α2, α-subunit loop 2; β1, β2, β3, β-subunit loops 1, 2, and 3, respectively; s/hCGβ, chimera of chum salmon FSH and hCG β-subunits; NH_2 terminus, amino-terminal end of the chum salmon β-subunit; SB, chum salmon seatbelt; TL, chum salmon seatbelt tensor loop; St, chum salmon seatbelt strap; tsFSH, teleost follitropin; LH, lutropin. The structures of the analogs used in this work can be determined by reference to Fig. 2.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

Seatbelt Latch Disulfide
Tensor Disulfide
Tensor Disulfide
Seatbelt Latch Disulfide
hCG
Salmon FSH

FIG. 1. **Comparison of the structures of hCG and salmon FSH showing the difference in the sizes of the holes in the β-subunits when the seatbelt is latched to β1 and the NH_2 terminus.** The Cα carbons of hCG (*left panel*) and a model of salmon FSH (*right panel*) are shown here. Atoms of β-subunit residues in the subunit core and seatbelt that surround α2 are highlighted. Note that the size of this hole appears to be considerably greater when the seatbelt is latched to a cysteine in β1 than when it is latched to a cysteine in the NH_2 terminus. The smaller size of the β-subunit hole in salmon FSH would make it more difficult to thread loop α2 beneath the seatbelt, a phenomenon that would account for its greater acid stability than hCG (10, 11). Therefore, one might expect that the assembly of salmon FSH differs from that of hCG. Color code: *white*, α-subunit; *dark gray*, β-subunit; *hatched patches*, β-subunit hole straddled by loop α2.

Human α-Subunit:

•α-Q5C •α-L41C •α-s43C

APDVQDCPECTLQENPFFSQPGAPILQCMGCCFSRAYPTPLRSKKTMLVQ

α-Subunit N-Terminus — Loop α1 — Loop α2

•α-S92CC

KNVTSESTCCVAKSYNRVTVMGGFKVENHTACHCSTCYYHKS

Loop α3 — α-Subunit C-Terminus

hCG β-Subunit Analogs And Salmon FSH

•C_5 (L5C)
•GTEC_5RYG (Nt) •A_{26} (C26A)

SKEPL$_5$RPRCRPINATLAVEKEGCPVC$_{26}$ITVNTTICAGYCPTMTRVLQG

β-Subunit N-Terminus — Loop β1 — Loop β2

VLPALPQVVCNYRDVRFESIRLPGCPRGVNPVVSYAVALSCQC

Loop β2 — Loop β3

•DRISMATPSC$_{110}$IV (St)
•KTDNTD(TL)
•KTDNTDC$_{100}$DRISMATPSC$_{110}$IV (SB)
•KC$_{93}$KTDNTDC$_{100}$DRISMATPSC$_{110}$IV (L92K,SB)

ALC$_{93}$RRSTTDC$_{100}$GGPKDHPLTC$_{110}$DDPRFQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ

Seatbelt — β-Subunit C-Terminus

FIG. 2. **Sequences of the α- and β-subunit analogs used in these studies.** Cysteines were substituted for residues in the α-subunit to create analogs whose names are indicated at the sites of the mutations. Changes to the hCG β-subunit are indicated *above* the hCG β-subunit sequence, which is *boxed*. The name of each mutation described in the text is indicated in *parentheses* following its sequence. For example, C26A indicates that cysteine residue 26 in loop β1 had converted to alanine; *Nt* indicates that the hCG β-subunit NH_2-terminal sequence Lys-Glu-Pro-Leu-Arg-Pro-Arg had been replaced by the salmon NH_2-terminal sequence Gly-Thr-Glu-Cys^4-Arg-Tyr-Gly. Note that residues Cys^{26} and Cys^5 are seatbelt latch sites in β1 and NH_2 terminus, respectively. Some analogs are combinations of these mutations as suggested by their names. Thus, s/hCGβ-Nt,C26A,SB is a salmon FSH and hCG β-subunit chimera with the hCG β-subunit NH_2-terminal and seatbelt sequences replaced with those from salmon FSH and the seatbelt latch site in loop β1 Cys^{26} replaced with alanine.

methods used in these studies have been described in the preceding accompanying articles (19, 20).

RESULTS

Some Heterodimers Containing β-Subunits That Are Chimeras of Salmon FSH and hCG Are Assembled Differently from hCG—To study the assembly of heterodimers that have the teFSH folding pattern, we employed an hCG β-subunit analog that contained the salmon FSH β-subunit NH_2 terminus and seatbelt. This β-subunit chimera, s/hCGβ-Nt,SB,C26A (Fig. 2), retained binding sites for most hCG monoclonal antibodies that recognize epitopes formed by the subunit core, which includes its cystine knot and loops β1, β2, and β3. Because this analog contains an alanine in place of βCys^{26}, the normal hCG β-subunit seatbelt latch site, it was expected to latch its seatbelt to βCys^5, a cysteine within its NH_2-terminal salmon sequence. This β-subunit chimera was incorporated into heterodimers that contained the native human α-subunit, but much less efficiently than hCGβ (Table I, data rows 1 and 7). Unlike the hCG heterodimer, which dissociated completely at pH 2 within 30 min at 37 °C, only about 20% of the hCG/teFSH chimera heterodimer dissociated under these conditions. It was not as stable as heterodimers that contain an intersubunit disulfide, which do not dissociate under these conditions (13). These observations showed that we could distinguish heterodimers in which the seatbelt is latched to the α-subunit, heterodimers in which the seatbelt is latched to βCys^5, and heterodimers in which the seatbelt is latched to βCys^{26} by measuring differences in their stabilities at pH 2, 37 °C. These differences become much more apparent following an overnight treatment at pH 2, 37 °C (Fig. 3).

The hCGβ and s/hCGβ-Nt,SB,C26A subunits differ in their NH_2-terminal ends, their seatbelts, and in loop β1. Studies described next were designed to learn how each of these regions influence heterodimer assembly. Replacing hCG β-subunit residue βLeu^5 with cysteine created hCGβ-L5C, an hCG analog that has a seatbelt latch site in an NH_2-terminal location corresponding to that in salmon FSH. Heterodimers that contained the native human α-subunit and hCGβ-L5C dissociated rapidly at pH 2, 37 °C, and were recognized by B111 (Table I, row 2), indicating that their seatbelts were latched to βCys^{26} in loop β1, not to βCys^5. Thus, introduction of a potential latch site into the hCGβ NH_2 terminus was not sufficient to alter the manner in which the heterodimer is assembled. In contrast, elimination of the seatbelt latch site in loop β1 of hCGβ-L5C reduced its ability to form heterodimers with the native human α-subunit as can be seen by the lack of heterodimer formed when the α-subunit was co-expressed with hCGβ-L5C,C26A (Table I, row 3). This finding is consistent with the notion that the hCG seatbelt of hCGβ-L5C,C26A became latched to βCys^5 prior to subunit docking and/or that the seatbelt did not become latched to βCys^5 after the subunits had docked. The propensity of the hCG seatbelt to be latched prior to subunit docking (19) suggests that it may have become latched to this site prior to the start of assembly. This would indicate that it can interfere with threading when latched to a cysteine in the NH_2 terminus.

To learn how the salmon FSHβ NH_2 terminus affected assembly, we expressed s/hCGβ-Nt and s/hCGβ-Nt,C26A with the native human α-subunit (Table I, rows 4 and 5). The presence of the salmon FSHβ NH_2 terminus appeared not to affect most heterodimer assembly. Consequently, 90% of the het-

The Journal of Biological Chemistry
ASBMB
jbc ONLINE
Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE I
Relative influence of the β1 and N-terminal latch sites on heterodimer assembly

COS-7 cells were transfected with either the native human α-subunit and hCGβ or the indicated analog.

Data row	Analog transfected: α-Subunit	Analog transfected: β-Subunit	Total dimer[a]	Dimer detected by B111[b]	Acid-stable dimer[c]	Probable seatbelt latch disulfide
				(ng/50μl ± SEM)[2]		
1	Native	hCGβ	6.72 ± 0.36	6.95 ± 0.26	<0.1	$\beta Cys^{110}/\beta Cys^{26}$
2	Native	hCGβ-L5C	7.32 ± 0.52	4.81 ± 0.18	<0.1	$\beta Cys^{110}/\beta Cys^{26}$
3	Native	hCGβ-L5C,C26A	<0.1	<0.1	<0.1	$\beta Cys^{110}/\beta 5$
4	Native	s/hCGβ-Nt	5.23 ± 0.20	6.37 ± 0.13	0.42 ± 0.08	$\beta Cys^{110}/\beta C26\beta Cys^{5}$
5	Native	s/hCGβ-Nt,C26A	<0.1	<0.1	<0.1	$\beta Cys^{110}/\beta 5$[d]
6	Native	s/hCGβ-Nt,SB	0.16 ± 0.01	<0.1	0.12 ± 0.03	$\beta Cys^{110}/\beta 5$[e]
7	Native	s/hCGβ-Nt,SB,C26A	0.91 ± 0.06	<0.1	0.74 ± 0.09	$\beta Cys^{110}/\beta 5$

[a] Heterodimer secreted into the culture medium was determined in A113/^{125}I-B110 sandwich assays.
[b] Latching of the seatbelt to β1 was determined in A113/^{125}I-B111 sandwich assays. B111 does not recognize the salmon FSH seatbelt when it is latched to βCys^{26}, however.
[c] The acid stability of the heterodimer was determined in A113/^{125}I-B110 sandwich assays of media treated at pH 2 for 30 min at 37 °C.
[d] Because we were unable to detect any heterodimer, we assumed the seatbelts of these analogs were either unlatched or, more likely, became latched to the cysteine in the N_2-terminal end of the β-subunit prior to formation of the heterodimer.
[e] The seatbelt was assumed to be latched to the NH_2-terminal end of the β-subunit based on the finding that the small amounts of heterodimer formed are stable at low pH. We expected that the seatbelt was latched to βCys^{26} in most of the free β-subunit but this could not be determined using existing antibodies due to the fact that B111 does not recognize the salmon seatbelt, even when it is latched to βCys^{26}.

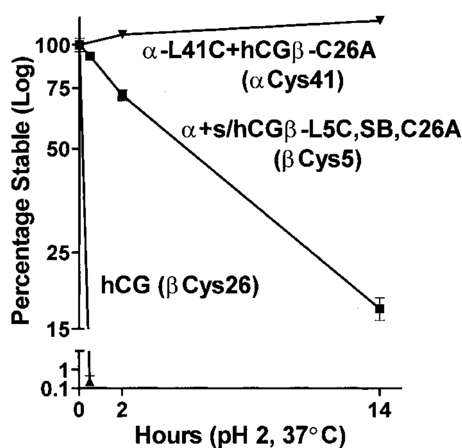

FIG. 3. **Variation in heterodimer stability as a function of the seatbelt latch site.** The acid stabilities of heterodimers in which the seatbelt is latched to βCys^{26} (*i.e.* hCG), βCys^{5} (*i.e.* α+s/hCGβ-L5C,SB,C26A), and α-subunit residue αCys41 (*i.e.* α-L41C+hCGβ-C26A) were determined at pH 2, 37 °C. The heterodimer containing the native hCG β-subunit dissociated completely within 30 min. That in which the seatbelt is latched to the α-subunit did not dissociate during an overnight incubation. The heterodimer in which the seatbelt was latched to βCys^{5} had a half-life of ~5–6 h.

erodimers containing s/hCGβ-Nt dissociated within 30 min at pH 2, 37 °C, and were readily recognized by antibody B111 (Table I, row 4). The latter observation showed that their seatbelts were latched to βCys^{26} in loop β1. The remaining 10% survived pH 2 treatment, 37 °C for 30 min, however, indicating that their seatbelts were latched to βCys^{5} in the salmon FSHβ NH_2 terminus. Preventing s/hCGβ-Nt from latching its seatbelt to loop β1 by converting βCys^{26} to alanine inhibited heterodimer assembly as seen by the lack of incorporation of s/hCGβ-Nt,C26A into heterodimers (Table I, row 5). Considered together, these observations suggested that the hCG seatbelt present in either s/hCGβ-Nt or s/hCGβ-Nt,C26A can become latched to βCys^{5} in the salmon FSH NH_2 terminus before or after the subunits dock; latching it to βCys^{5} prior to subunit docking appeared to inhibit assembly.

The teFSH seatbelt has a much greater influence than the NH_2-terminal latch site on heterodimer assembly. Much less heterodimer was formed when the native human α-subunit was co-expressed with an analog of s/hCGβ-Nt in which the hCG seatbelt was replaced with the salmon seatbelt (*i.e.* s/hCGβ-Nt,SB, Table I, row 6). The small amount of s/hCGβ-Nt,SB that was assembled into heterodimers containing the native α-subunit was roughly as stable as that containing s/hCGβ-Nt,SB,C26A, suggesting that its seatbelt was latched to βCys^{5} in the NH_2 terminus and not to βCys^{26} in loop β1. In fact, heterodimers containing s/hCGβ-Nt,SB,C26A, a β-subunit analog that has only a single seatbelt latch site, were produced more efficiently than those containing s/hCGβ-Nt,SB (Table I, data rows 6 and 7). This observation indicated that the salmon FSH seatbelt in s/hCGβ-Nt,SB became latched to each of these potential latch sites and that it may block threading when it is latched to βCys^{26}. In subsequent studies, we converted βCys^{5} of s/hCGβ-Nt,SB to alanine, thereby creating s/hCGβ-Nt,SB,C5A, which lacks an NH_2-terminal seatbelt latch site. This abolished heterodimer formation (not shown), confirming the notion that the seatbelt in heterodimers containing s/hCGβ-Nt,SB is latched to βCys^{5} and that latching the seatbelt to βCys^{26} in loop β1 suppressed assembly.

Assembly of Glycoprotein Hormones in Which the Seatbelt Is Latched to a Cysteine in the β-Subunit NH_2 Terminus Occurs by a Wraparound Route—Heterodimers in which the seatbelt was latched to the β-subunit NH_2 terminus dissociated at pH 2, 37 °C, albeit much slower than hCG (Fig. 3). This showed that during acid-induced dissociation of teFSH analogs the glycosylated end of loop α2 can pass through the space between the seatbelt and the β-subunit core. Theoretically, the reversal of this process would permit heterodimer assembly by a threading mechanism, albeit at a very slow rate. The small space between the seatbelt and the subunit core suggested that threading would be highly unlikely, even if the tensor disulfide were to be disrupted during threading as is the case during the assembly of hCG (20). The threading and wrapping pathways could be distinguished if we were able to determine whether the seatbelt had been latched before or after the subunits docked. As discussed next, this can be accomplished using disulfide cross-links to trap early assembly intermediates.

To trap early docking intermediates, we took advantage of the fact that the NH_2-terminal ends of the subunits become adjacent when the heterodimer is assembled (2, 3). We reasoned that converting αGln^{5} to cysteine to create α-Q5C would enable the formation of an α5-β5 intersubunit disulfide, but only if the subunits docked before the seatbelt was latched (Fig. 4). Furthermore, because αCys^{5} is located near βCys^{5}, the seatbelt latch site, we expected that during the wraparound pathway the seatbelt might also be latched to αCys^{5} instead of βCys^{5}, provided the α5-β5 disulfide had not already been formed. Either of these phenomena would have resulted in an

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

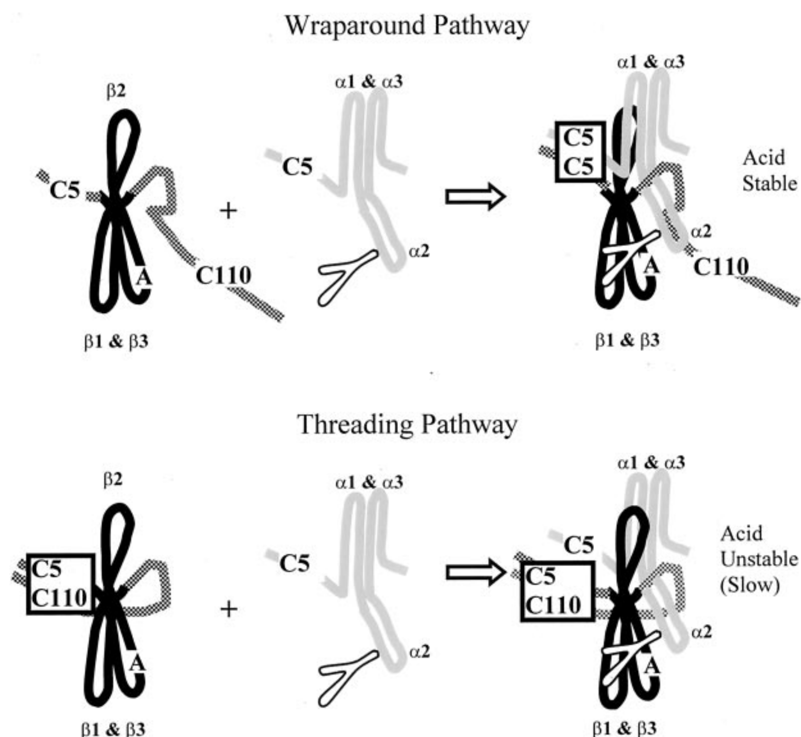

FIG. 4. **The mechanism of teFSH assembly can be distinguished by monitoring the assembly of heterodimers that contain α-Q5C.** *Top row*, during assembly of teFSH analogs by a wraparound mechanism, the β-subunit docks with the α-subunit before the seatbelt is latched. This will position αCys^5 adjacent to βCys^5, with which it is likely to form an intersubunit disulfide. This will make the resulting heterodimer completely resistant to dissociation at pH 2, 37 °C. *Bottom row*, during assembly of these analogs by a threading mechanism, the seatbelt becomes latched to βCys^5 before the subunits dock. This would prevent the formation of an intersubunit αCys^5-βCys^5 disulfide and the heterodimer will have a stability similar to that of teFSH. Consequently, the heterodimer will dissociate with a half-life of ~5–6 h at pH 2, 37 °C.

intersubunit disulfide cross-link that would prevent the heterodimer from dissociating upon treatment overnight at pH 2, 37 °C. In contrast, if assembly occurs by a threading mechanism instead of a wraparound mechanism, seatbelt residue βCys^{110} would have been cross-linked to βCys^5 prior to docking. This would have prevented the formation of both the α5-β5 and α5-β110 disulfides and led to the formation of a heterodimer that dissociated with a half-life of 5–6 h during treatment at pH 2, 37 °C. Of course, it was also possible that during wrapping, seatbelt residue βCys^{110} would become latched to βCys^5 to create a heterodimer that would also dissociate with a half-life of 5–6 h. Therefore, a finding that all the heterodimer dissociated with a half-life of 5–6 h would indicate but not prove that assembly occurs by a threading mechanism. However, the finding that a substantial fraction of the heterodimer was acid stable would show that most, if not all, heterodimer assembly had occurred by a wraparound mechanism.

There was one other caveat to this approach. Co-expression of α-Q5C and hCGβ-R8C, analogs in which cysteines are substituted for hCG residues αGln^5 and βArg^8 has been shown to form a disulfide cross-linked heterodimer (14). Formation of this disulfide is explained by the distances between the Cα and Cβ atoms of these residues (*i.e.* roughly 5.2 and 3.9 Å), which are similar to those in typical glycoprotein hormone disulfides (*i.e.* roughly 5–6.5 and 4 Å, respectively). The location of the salmon FSH β-subunit latch site corresponds to hCG β-subunit residue βLeu^5, not βArg^8, however. The distances between the Cα and Cβ atoms of αGln^5 and βLeu^5 are ~9.1 and 11.1 Å, suggesting that when these residues are replaced with cysteines, their positions in the heterodimer might prevent them from forming a disulfide.

To learn if an α5-β5 disulfide bridge can form during heterodimer assembly, we co-expressed hCGβ-L5C with the native α-subunit and with α-Q5C. Heterodimers formed with the native α-subunit were unstable at acid pH and dissociated within 30 min (Table I, data row 2); those formed when hCGβ-L5C was co-expressed with α-Q5C remained intact following an overnight incubation at pH 2, 37 °C (Table II, data row 1). Both heterodimers were recognized by antibody B111 (not shown), demonstrating that their seatbelts were latched to residue βCys^{26}. This showed that heterodimers containing α-Q5C and hCGβ-L5C are stabilized by an α5-β5 disulfide bridge.

We reasoned that if seatbelt residue βCys^{110} is latched to βCys^5 before the teFSH β-subunit docks with α-Q5C, neither of these β-subunit cysteines can be cross-linked to αCys^5. We tested this possibility by co-expressing α-Q5C with hCGβ-L5C,C26A, an analog that has a teFSH folding pattern and in which the seatbelt appears to be latched efficiently to βCys^5 before the subunits dock. This analog did not form detectable amounts of heterodimer with the native α-subunit (Table I, data row 3) and only small amounts were incorporated into heterodimers containing α-Q5C (Table II, row 2). Apparently, the seatbelt of hCGβ-L5C,C26A became latched to βCys^5 before the subunits could dock, which precluded the formation of an αCys^5-βCys^5 disulfide bridge. The finding that small amounts of hCGβ-L5C,C26A were incorporated into heterodimers containing α-Q5C (Table II, row 2) showed that α-Q5C can be used to trap β-subunits that contain unlatched seatbelts, even when these represent a small fraction of the total β-subunit population. Based on the finding that less than 10% of these heterodimers dissociated after an overnight incubation at pH 2, 37 °C (Table II, data row 2), we anticipate that 9 of 10 heterodimer molecules contained an intersubunit disulfide between αCys^5 and βCys^5 or βCys^{110}, which indicates that the subunits docked before the seatbelts were latched and that they were formed by a wraparound mechanism. The remaining heterodimer molecules appeared to contain an intrasubunit disulfide between βCys^5 and βCys^{110}. Whereas these could have been formed by a threading mechanism, the finding that the vast majority of heterodimers containing hCGβ-L5C,C26A were assembled by a wraparound mechanism suggested that the β5-β110 disulfide had also been formed by a wraparound mechanism after the subunits dock. As expected from the fact that hCGβ-L5C,C26A lacks a loop β1 latch site, heterodimers containing α-Q5C and hCGβ-L5C,C26A were not detected by B111 (not shown).

These findings showed that the pathway used to assemble heterodimers containing teFSH analogs can be deduced from

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE II

The ability of αCys[5] to compete for seatbelt for the β-subunit N-terminal latch site

COS-7 cells were transfected with α-Q5C and hCGβ analogs with their NH_2-terminal and/or seatbelt sequences replaced by the counterparts of their salmon FSH counterparts as indicated. The loop β1 seatbelt latch site was also removed in some analogs by replacing βCys[26] with alanine. Heterodimer secreted into the culture medium was quantified in A113/^{125}I-B110 sandwich assays. The stabilities of the heterodimers were determined in A113/^{125}I-B110 sandwich assays of media treated at pH 2 for 30 min or overnight at 37 °C. All values are mean ± S.E. of triplicate transfections.

Data row	Analog	Total dimer	pH 2, 0.5 hours	pH 2, 16 hours
		ng/50μl ± S.E.	*% total ± S.E.*	
1	α-Q5C + hCGβ-L5C	20.02 ± 1.94	102.1 ± 6.1	100.3 ± 3.6
2	α-Q5C + hCGβ-L5C,C26A	1.12 ± 0.07	92.7 ± 0.7	91.9 ± 1.8
3	α-Q5C + s/hCGβ-Nt	7.25 ± 0.09	97.1 ± 1.5	102.9 ± 2.5
4	α-Q5C + s/hCGβ-Nt,C26A	0.73 ± 0.10	91.9 ± 3.7	86.0 ± 3.5
5	α-Q5C + s/hCGβ-Nt,SB,C26A	1.98 ± 0.05	89.9 ± 1.0	89.0 ± 0.9
6	α-Q5C + s/hCGβ-L5C,SB,C26A	8.22 ± 0.12	104.1 ± 2.8	102.7 ± 0.9

the abilities of their NH_2-terminal β-subunit cysteines to become cross-linked to the NH_2-terminal cysteine of α-Q5C. To learn how the salmon NH_2 terminus affected subunit docking and seatbelt latching, we monitored the acid stabilities of heterodimers containing α-Q5C and s/hCGβ-Nt or s/hCGβ-Nt,C26A. These studies revealed that the pathway of assembly was essentially the same when the NH_2-terminal latch site was surrounded by hCG or salmon FSH β-subunit residues. Coexpression of α-Q5C with s/hCGβ,Nt led to a substantial amount of acid-stable heterodimer (Table II, row 3) that was detected readily by B111 (not shown). This revealed that the hCG seatbelt of s/hCGβ-Nt had been latched to βCys[26] in loop β1 prior to subunit docking, thereby enabling βCys[5] in the β-subunit NH_2 terminus to be cross-linked to αCys[5] in the α-subunit NH_2 terminus. In contrast, much smaller amounts of heterodimer were formed when βCys[26] was replaced with alanine to create s/hCGβ-Nt,C26A (Table II, row 4). The reduction in heterodimer assembly caused by this change showed that the hCG seatbelt in s/hCGβ-Nt,C26A had been latched to βCys[5] prior to assembly. In this position the seatbelt inhibited threading and, by being in a disulfide with βCys[5], prevented the formation of the α5-β8 disulfide. As had been observed for heterodimers containing α-Q5C and hCGβ-L5C,C26A, a small fraction of the heterodimers containing α-Q5C and hCGβ-Nt,C26A were acid labile. This indicated that some of the seatbelt had been latched to βCys[5], most likely after the subunits had docked.

To learn how the composition of the seatbelt affected subunit docking and seatbelt latching, we monitored the acid stabilities of heterodimers containing α-Q5C and β-subunits s/hCGβ-Nt,SB,C26A and s/hCGβ-L5C,SB,C26A. These β-subunits are analogs of s/hCGβ-Nt,C26A and hCGβ-L5C,C26A in which the hCG seatbelt has been replaced with its salmon FSH counterpart. The presence of the salmon seatbelt increased the amount of heterodimer formed significantly. For example, 3-fold more heterodimer was obtained with s/hCGβ-Nt,SB,C26A than with s/hCGβ-Nt,C26A (Table II, rows 5 and 4) and 7–8-fold more heterodimer was obtained with s/hCGβ-L5C,SB,C26A than with hCGβ-L5C,C26A (Table II, rows 6 and 2). Most of the heterodimers formed were acid stable, which is consistent with the notion that they had been formed by a wraparound mechanism. These findings indicated that the salmon FSH seatbelt is not latched to βCys[5] as rapidly as the hCG seatbelt and, as a result, more βCys[5] was available to form a disulfide with αCys[5]. As had been observed for heterodimers containing the hCGβ-L5C,C26A and s/hCGβ-Nt,C26A subunits, about 10% of the heterodimer containing the s/hCGβ-Nt,SB,C26A subunit dissociated at low pH. This suggested that the seatbelt had also become latched to βCys[5] in a small fraction of the heterodimer, most likely by a wraparound mechanism.

The teFSH Seatbelt Can Be Latched to the α-Subunit, an Observation That Confirms Assembly Occurs by a Wraparound Mechanism—The observation that αCys[5] became cross-linked to the β-subunit during the assembly of heterodimers containing α-Q5C and β-subunits having the teFSHβ folding pattern strongly supports the notion that these teFSH analogs are assembled by a wraparound mechanism. To test this result in an alternative fashion, we repeated these studies using α-subunit analogs having unpaired cysteines located at other sites (Table III). We have found that hCG seatbelt residue βCys[110] can be latched to cysteines added to the α-subunit when it is prevented from being latched to βCys[26] in loop β1 (13). To learn if the salmon FSH seatbelt behaved similarly to the hCG seatbelt in either regard, we compared the stabilities of heterodimers containing α-L41C or α-S43C and either s/hCGβ-Nt,SB,C26A or s/hCGβ-L5C,SB,C26A (Table III). Heterodimers containing the native α-subunit (Table III, data rows 3 and 6) were more stable than hCG (Table III, data row 1), but not nearly as stable as the cross-linked heterodimers that contained α-L41C and hCGβ-C26A in which the seatbelt is latched to αCys[41] (Table III, row 2) or heterodimers that contained cysteines added to parts of loop α2 (Table III, rows 4, 5, 7, and 8). This observation shows that cysteines added to the α-subunit compete with βCys[5] as a seatbelt latch site, confirming the notion that hCG analogs in which the seatbelt is latched to a cysteine in the NH_2-terminal end of the β-subunit are formed by a wraparound pathway. This suggests that all glycoprotein hormone analogs having the teFSH architecture are likely to be formed by a wraparound mechanism, not by threading.

These studies revealed that teFSHβ analogs containing the salmon FSH seatbelt and an hCGβ NH_2-terminal latch site were incorporated into heterodimers better than those in which both regions were derived from salmon FSHβ (Table III, data rows 3–5 and 6–8). This observation contradicted our expectations that the salmon NH_2 terminus would serve as the more efficient latch site. One explanation for this observation is that the salmon FSH seatbelt has a greater tendency to be latched to the salmon FSHβ NH_2 terminus before the subunits dock, a phenomenon that would reduce assembly. Another explanation is that residues at the NH_2-terminal end of the β-subunit have a role in subunit docking (15, 16). Those in hCGβ may be more effective in promoting subunit docking than those in salmon FSHβ.

The Composition of the Seatbelt Can Influence Threading—Differences in the assembly of heterodimers containing s/hCGβ-Nt and s/hCGβ-Nt,SB indicated that the composition of the seatbelt might have a substantial influence on the mechanism of heterodimer assembly. Although each of these β-subunits has two potential seatbelt latch sites, the hCG seatbelt of s/hCGβ-Nt became latched to βCys[26] and permitted assembly by a threading mechanism. As had been seen earlier (Table I, data row 6), assembly of heterodimers containing the native

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

The Journal of Biological Chemistry

ASBMB

jbc ONLINE

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE III

Cysteines added to the α-subunit compete with that at βCys^{5} for latching the seatbelt

Heterodimers secreted into the culture medium by COS-7 cells co-transfected in triplicate with the indicated α- and β-subunits were concentrated 10-fold and quantified in A113/^{125}I-B110 assays. The samples were then treated at pH 2, at 37 °C for 30 min, 120 min, and overnight before being assayed a second time. Values shown (mean ± S.E. for triplicates) represent the amount of material in the concentrated medium and the percentage that remained as heterodimer following acid treatment. The least acid-stable heterodimer is hCG, in which the seatbelt is latched to βCys^{26} in loop β1. The stability of α-L41C/hCGβ-C26A is typical of heterodimers in which the seatbelt is latched to a cysteine in the α-subunit. The stabilities of heterodimers having the seatbelt latched to βCys^{5} (*i.e.* those containing the native α-subunit and s/hCGβ-Nt,SB,C26A, s/hCGβ-L5C,SB,C26A, s/hCGβ-Nt,SB, or hCGβ-L5C,C26A-KDEL) is between that of hCG and α-L41C/hCGβ-C26A. The increased stability of chimeras containing α-subunit analogs having an additional cysteine is due to the presence of an intersubunit disulfide. Formation of this cross-link can occur only if the seatbelt is latched by a wraparound mechanism. (Note that in two other experiments, we observed that 15.2 and 19.2% of the s/hCGβ-L5C,SB,C26A containing heterodimer was stable after overnight incubation, values that indicate that the heterodimers containing the native human α-subunit and s/hCGβ-L5C,SB,C26A or s/hCGβ-Nt,SB,C26A have approximately the same stability.)

Data row	α-Subunit analog	β-Subunit analog	Total dimer	Remaining pH 2, 30 min	Remaining pH 2, 120 min	Remaining pH 2, 16 h	Probable seatbelt latch site
			ng/50 μl		*% total ± S.E.*		
1	Native	hCGβ	28.81 ± 0.05	1.4 ± 0.1	0.9 ± 0.1	1.0 ± 0.0	β110:β26
2	α-L41C	hCGβ-C26A	14.81 ± 0.18	93.0 ± 7.1	107.1 ± 0.6	117.8 ± 4.9	β110:α41
3	Native	s/hCGβ-Nt,SB,C26A	5.11 ± 0.06	53.7 ± 1.0	35.1 ± 1.2	22.3 ± 1.7	β110:β5
4	α-L41C	s/hCGβ-Nt,SB,C26A	2.66 ± 0.01	61.7 ± 0.6	58.9 ± 1.7	62.8 ± 4.3	β110:α41>β5
5	α-S43C	s/hCGβ-Nt,SB,C26A	3.94 ± 0.15	77.1 ± 0.6	68.9 ± 3.3	70.8 ± 1.7	β110:α43>β5
6	Native	s/hCGβ-L5C,SB,C26A	16.60 ± 0.60	109.9 ± 1.3	84.5 ± 0.6	34.2 ± 1.1	β110:β5
7	α-L41C	s/hCGβ-L5C,SB,C26A	18.01 ± 2.17	86.7 ± 0.6	72.3 ± 2.3	76.0 ± 0.3	β110:α41>β5
8	α-S43C	s/hCGβ-L5C,SB,C26A	16.16 ± 0.17	88.9 ± 0.3	70.3 ± 2.7	113.3 ± 1.3	β110:α43>β5

α-subunit and s/hCGβ-Nt,SB was very inefficient (Table IV, row 3). Both s/hCGβ-Nt and s/hCGβ-Nt,SB were incorporated efficiently into heterodimers that contain α-Q5C (Table IV, row 1 and 2), showing that their seatbelts were latched primarily to loop β1 residue βCys^{26} prior to subunit docking. The difference in the amount of heterodimer formed when s/hCGβ-Nt,SB was expressed with the native α-subunit and α-Q5C suggested that the salmon FSH seatbelt inhibited threading when it was latched to βCys^{26}. Studies described next were initiated to test this notion and to identify the portion of the seatbelt that was most likely to be responsible for its ability to interfere with threading.

We monitored the formation of heterodimers containing the native α-subunit and s/hCGβ-SB or s/hCGβ-SB,L92K; the former seatbelt has two hCG residues at its NH_2 terminus, namely βAla^{91} and βLeu^{92}. In the chum salmon FSH seatbelt, these residues are isoleucine and lysine and, to learn how a positively charged residue adjacent to the tensor loop would affect assembly, we replaced βLeu^{92} with lysine. The amount of heterodimer made when either of these β-subunit chimeras was co-expressed with the native α-subunit was much lower than that of hCG (Table V, data rows 1–3). This showed that the presence of the salmon FSH seatbelt reduced assembly, most likely by impeding threading.

The NH_2- and COOH-terminal halves of the hCG and salmon FSH seatbelts differ significantly and we studied each of these regions separately. The NH_2-terminal half of the seatbelt contains a small loop that is stabilized by a disulfide that we term the "tensor" because of its ability to change the length of the seatbelt during heterodimer assembly. The tensor disulfide is disrupted during threading, which elongates the seatbelt and facilitates passage of the glycosylated end of loop α2 through the β-subunit (20). Reformation of the tensor disulfide following threading stabilizes the heterodimer. To test the notion that differences in the stabilities of the hCG and salmon FSH tensor loops were responsible for the reduced ability of the salmon FSH seatbelt to permit threading, we replaced the hCG tensor loop with its salmon FSH counterpart and monitored heterodimer assembly. The salmon FSH tensor loop had little or no inhibitory influence on heterodimer assembly. Consequently, s/hCGβ-TL was incorporated into heterodimers as well as or better than hCGβ and much better than s/hCGβ-SB or s/hCGβ-SB,L92K, analogs that have nearly the entire salmon FSH seatbelt (Table V, data rows 1–4).

The COOH-terminal half of the seatbelt constrains the position of loop α2 but makes few specific contacts with this portion of the α-subunit needed to stabilize the heterodimer. Furthermore, the position of this part of the seatbelt depends primarily on the location of the seatbelt latch site, which is found in loop β1 of most vertebrate hormones and in the β-subunit NH_2 terminus in salmon FSH (1). This can be varied experimentally in hCG analogs by moving the seatbelt latch site to different parts of the α-subunit (13). Because this portion of the hCG seatbelt appears to contribute little to lutropin activity other than to keep the heterodimer intact, we refer to it as the "strap." The strap region has been shown to have a dramatic influence on FSH and TSH activity, however, even though the mechanism of this phenomenon remains unknown (6, 8, 17). Substitution of the hCG strap with its salmon FSH counterpart reduced heterodimer assembly dramatically (Table V, data row 5). This showed that the strap region of the salmon FSH seatbelt is responsible for its ability to suppress threading when it is latched to βCys^{26}. Heterodimers that contain this portion of the seatbelt dissociate rapidly at pH 2, 37 °C, indicating that when the strap is latched to βCys^{26} in loop β1 it does not block threading during acid-induced heterodimer dissociation.

To learn if heterodimers in which the salmon FSH seatbelt strap regions were latched to βCys^{26} had been formed by threading or wraparound mechanisms, we expressed s/hCGβ-St and s/hCGβ-St,C26A with α-S43C and α-S92C. Previously, we had found that the hCG seatbelt in hCGβ-C26A is latched readily by a wraparound mechanism to either of these α-subunit analogs when it is prevented from latching with βCys^{26} (13). By analogy, we reasoned that if heterodimers containing the salmon seatbelt strap were latched by a wraparound mechanism, s/hCGβ-St,C26A would be latched readily to both α-S43C and α-S92C. Co-expression of s/hCGβ-St,C26A with either α-subunit analog resulted in the formation of only trace amounts of heterodimer (not shown). This suggested that chimeras containing the native α-subunit and the salmon FSH seatbelt strap are more likely to form by a threading mechanism than a wraparound mechanism. The finding that only small quantities of heterodimer are formed suggested that threading is very inefficient, most likely because the strap region interfered with passage of loop α2 beneath the seatbelt.

We reasoned that if the salmon strap reduced the rate of threading relative to that seen during hCG assembly, it would facilitate the formation of a disulfide cross-link between a cys-

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 34, Issue of August 20, pp. 35458–35468, 2004
© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

IV. PROBABLE MECHANISM OF SUBUNIT DOCKING AND COMPLETION OF ASSEMBLY*

Received for publication, March 18, 2004, and in revised form, May 14, 2004
Published, JBC Papers in Press, May 27, 2004, DOI 10.1074/jbc.M403055200

Yongna Xing, Rebecca V. Myers, Donghui Cao, Win Lin, Mei Jiang, Michael P. Bernard, and William R. Moyle‡

From the Department of Obstetrics and Gynecology, University of Medicine and Dentistry of New Jersey, Robert Wood Johnson (Rutgers) Medical School, Piscataway, New Jersey 08854

The unique structures of human choriogonadotropin (hCG) and related glycoprotein hormones make them well suited for studies of protein folding in the endoplasmic reticulum. hCG is stabilized by a strand of its β-subunit that has been likened to a "seatbelt" because it surrounds α-subunit loop 2 and its end is "latched" by an intrasubunit disulfide bond to the β-subunit core. As shown here, assembly begins when parts of the NH_2 terminus, cysteine knot, and loops 1 and 3 of the α-subunit dock reversibly with parts of the NH_2 terminus, cystine knot, and loop 2 of the hCG β-subunit. Whereas the seatbelt can contribute to the stability of the docked subunit complex, it interferes with docking and/or destabilizes the docked complex when it is unlatched. This explains why most hCG is assembled by threading the glycosylated end of α-subunit loop 2 beneath the latched seatbelt rather than by wrapping the unlatched seatbelt around this loop. hCG assembly appears to be limited by the need to disrupt the disulfide that stabilizes the small seatbelt loop prior to threading. We postulate that assembly depends on a "zipper-like" sequential formation of intersubunit and intrasubunit hydrogen bonds between backbone atoms of several residues in the β-subunit cystine knot, α-subunit loop 2, and the small seatbelt loop. The resulting intersubunit β-sheet enhances the stability of the seatbelt loop disulfide, which shortens the seatbelt and secures the heterodimer. Formation of this disulfide also explains the ability of the seatbelt loop to facilitate latching during assembly by the wraparound pathway.

Gonadotropins and thyrotropins are structurally related glycoprotein hormone heterodimers in which a loop of their α-subunits is surrounded by a strand of their β-subunits like a "seatbelt" (1–3). With the exception of some teleost fish follitropins, the seatbelts of most vertebrate hormones is "latched" by an intrasubunit disulfide to a cysteine in the β-subunit core. The unusual structures of these heterodimers and the fact that their assembly can be studied within cells makes them useful for identifying factors that affect protein folding in the ER[1] (4, 17–19). Assembly of most hCG, hFSH, and hTSH in the ER occurs after the seatbelt is latched by a process in which the glycosylated end of loop α2 is "threaded" through a hole in the β-subunit (17). This process is facilitated by disruption of a disulfide that we have termed the "tensor" because it stabilizes a small loop within the seatbelt that regulates its length (18). Disruption of the tensor disulfide prior to assembly enlarges the hole in the β-subunit and facilitates threading; reformation of the tensor disulfide following threading tightens the seatbelt around loop α2, which stabilizes the heterodimer. Alternatively, the hCG heterodimer can be assembled by a process in which the seatbelt is wrapped around loop α2 before the seatbelt latch disulfide is formed; formation of the seatbelt latch disulfide completes assembly. The "wrapping" mechanism, which was first proposed on the basis of pulse-chase analysis (4), appears to be used infrequently relative to the threading pathway for hCG assembly. Wrapping is required for assembly of hCG analogs in which the seatbelt is latched to a cysteine in the NH_2-terminal end of the β-subunit, a site comparable with that of the FSH β-subunit found in salmon and many other teleost fish (19). It is also required for the assembly of heterodimers in which the seatbelt is latched to the α-subunit (5).

Studies described here were designed to learn why most hCG is assembled by a threading mechanism rather than a wraparound mechanism. Conceivably, the latched seatbelt is a component of the subunit docking site. As a result, formation of the seatbelt latch disulfide would increase the affinity of the β-subunit for the α-subunit, thereby facilitating assembly by a threading route. Alternatively, the unlatched hCG seatbelt might occupy positions near the subunit interface where it would be in a position to interfere with subunit docking or where it can destabilize the docked complex. This would reduce the amount of docked complex and interfere with assembly by a wraparound route. The finding that the end of the seatbelt scans the β-subunit to find its latch site (18) supports the notion that the seatbelt could contact the subunit interface. Efforts to distinguish these possibilities led us to study how the hCG α- and β-subunits dock and to determine how the latching of the seatbelt affected this process.

Using intersubunit disulfide bonds to stabilize partially assembled intermediates, we found that the hCG β-subunit docks with the α-subunit in similar but not identical fashions when its seatbelt is latched or unlatched. Most docked complexes appear to dissociate before the heterodimer can be assembled by either pathway, a phenomenon that would favor threading by providing more time for the β-subunit to latch its seatbelt. Furthermore, the unlatched seatbelt appears to hinder docking and/or promote dissociation of the docked complex, a phenom-

* This work was supported by NICHD National Institutes of Health Grants HD14907 and HD38547. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 732-235-4224; Fax: 732-235-4225; E-mail: moyle@umdnj.edu.

[1] The abbreviations used are: ER, endoplasmic reticulum; α1, α2, α3, α-subunit loops 1, 2, 3; β1, β2, β3, β-subunit loops 1, 2, 3; hCG, human choriogonadotropin; hFSH, human follitropin; hTSH, human thyrotropin; teFSH, teleost FSH found in salmon and related species in which the seatbelt is latched to a cysteine in the NH_2-terminal end of the β-subunit. Abbreviations for all analogs are described in Fig. 1.

This paper is available on line at http://www.jbc.org

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

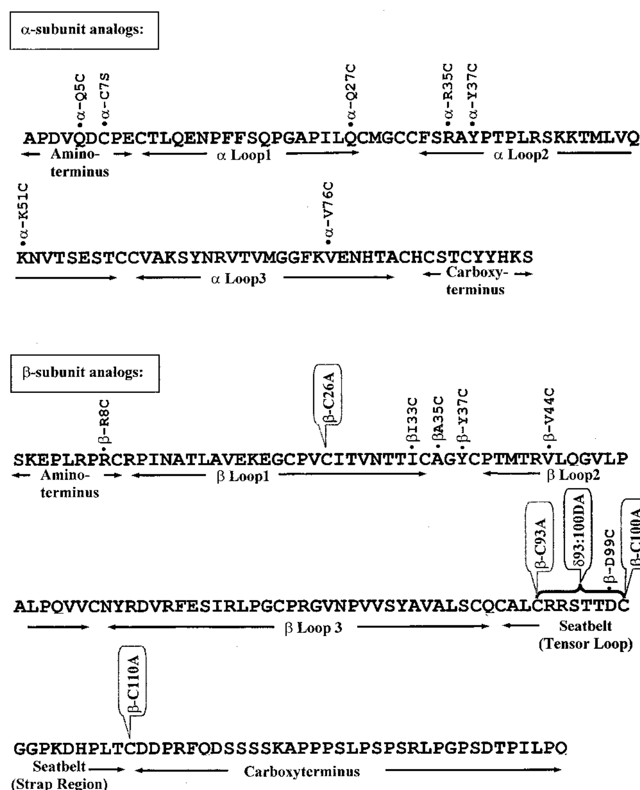

FIG. 1. **Constructs used in these studies.** The amino acid sequences of the constructs used in these studies are shown here. Residues *above* the sequence indicate amino acid substitutions. For example, α-Q5C represents an analog in which α-subunit residue αGln^{5} is converted to cysteine. Several analogs contain two or more substitutions. For example, *hCGβ-C26A,C110A* represents an analog in which both βCys^{26} and βCys^{110} are converted to alanine. As indicated by the *bracket*, *hCGβ-δ(93:100)DA* represents an analog in which all the residues in the tensor loop are replaced by aspartic acid and alanine. *hCGβ-δ(93:100)DA,C26A* is an analog of hCGβ-δ(93:100)DA in which βCys^{26} is converted to alanine, a mutation that prevents it from latching its seatbelt to loop β1. *δ1,7hCGβ* refers to an analog lacking residues 1–7 and that has an arginine at its N terminus (*i.e.* corresponding to hCG βArg^{8}). *δ2,8hCGβ* is a construct encoding an hCGβ analog missing residues 2–8. This analog has a serine at its NH_2 terminus (*i.e.* corresponding to hCG βSer^{1}).

enon that would also favor threading. By comparing the apparent positions of the subunits in the docked complexes with the structures of the assembled heterodimers, we devised models of heterodimer assembly. These suggest that while both threading and wrapping depend on the formation of similar intrasubunit and intersubunit hydrogen bonds, these would appear to form more readily by threading when the seatbelt is latched than by wrapping when it is unlatched.

EXPERIMENTAL PROCEDURES

Constructs used in these studies are illustrated in Fig. 1 and were produced by standard methods of site-directed mutagenesis (17). Methods used to transfect COS-7 cells and immunological procedures employed to measure the resulting heterodimers have also been described (17). To facilitate identification of the analogs used in each of the studies described here, we named them to reflect the hCG residues that have been changed. For example, hCGβ-R8C,C93A,C100A represents an analog of the hCG β-subunit in which codons for Arg^{8}, Cys^{93}, and Cys^{100} were replaced with cysteine, alanine, and alanine, respectively. The relative locations of antibody binding sites used in the hormone sandwich immunoassays are illustrated by Xing *et al.* (17). Briefly, most heterodimers were captured to microtiter plates using an antibody (A113) to the α-subunit and detected using a radioiodinated antibody (B110 or B111) to the β-subunit. Molecular modeling and cartoon illustrations were prepared with the aid of the programs Sybyl (Tripos, St. Louis, MO) and Sculpt (MDL Information Systems, Inc., San Diego, CA).

RESULTS

The Subunits Dock in Similar but Not Identical Fashions When the Seatbelt Is Latched and Unlatched—We employed a disulfide scanning mutagenesis strategy to identify portions of the subunits that are likely to contact one another when the seatbelt is unlatched or when the tensor disulfide is disrupted. These are key intermediates in the wraparound and threading pathways, respectively (17). The analogs used were derivatives of β-subunits that cannot latch their seatbelts (*i.e.* hCGβ-C26A,C110A) or form the tensor disulfide (*i.e.* hCGβ-C93A,C100A). While neither was capable of being assembled into stable heterodimers with the native α-subunit (6), both were incorporated into heterodimers that are cross-linked by an intersubunit disulfide (Fig. 2). This property formed the basis of our strategy for identifying portions of the subunits likely to contact one another during assembly. We assumed that the ability of a disulfide to "rescue" complexes containing docked subunits that cannot otherwise be assembled into a heterodimer would be proportional to the time that its component cysteines are adjacent. Based on our expectation that contacts between the α-subunit and these β-subunit analogs during wrapping and threading would be at least somewhat similar to those in the heterodimer, we introduced cysteines into each subunit at sites that were capable of cross-linking the heterodimer. This approach may have caused us to overlook transient contacts that do not lead to assembly, but these were of lesser interest for these studies.

Several intersubunit disulfides have been used to cross-link the subunits of hCG (7, 8) and we selected a few others by measuring the distances between the Cα carbons and between the Cβ carbons of every residue in the α- and β-subunits (not shown). We assumed that the most favorable disulfides would be at positions in which the maximum distances between these atoms would be ~6–6.5 and 3.8–4.2 Å, respectively. Based on these considerations, we substituted cysteines for residues in the hCG α- and β-subunits to produce heterodimers having the potential to form the following disulfides: αQ5C-βR8C, αQ27C-βV44C, αV76C-βV44C, αC7S-βY37C, αR35C-βA35C, αY37C-βI33C, and αK51C-βD99C (Fig. 1). Note that the conversion of αCys^{7} to serine in α-C7S disrupted its ability to form an intrasubunit disulfide between α-subunit residues 7 and 31. The resulting free α-subunit cysteine (*i.e.* αCys^{31}) can form a disulfide with the cysteine introduced into the β-subunit in place of hCG-βTyr^{37} (8). As described next, each of these analogs formed cross-linked heterodimers that were stable at pH 2, 37 °C, a condition known to promote hCG dissociation (9). Formation of these disulfides in complexes lacking the abilities to latch their seatbelts or to stabilize the small seatbelt loop indicated that these portions of the subunits were also adjacent at some time during the period in which the subunits were docked to one another.

To learn how interactions between the NH_2-terminal portions of the subunits might affect subunit docking, we studied the formation of analogs that were cross-linked by the α5-β8 disulfide. Co-expression of α-Q5C and hCGβ-R8C led to the formation of an acid-stable heterodimer, indicating that it contained an intersubunit disulfide. When α-Q5C was co-expressed with hCGβ-R8C,C26A,C110A and hCGβ-R8C,C93A,C100A, β-subunits that cannot latch their seatbelts or form their tensor disulfides, respectively, we observed that 125 and 39% as much heterodimer was formed as that containing hCGβ-R8C (Fig. 2, *left* and *right*). This showed that an intersubunit disulfide had formed between the NH_2-terminal ends of the α- and β-subunits while the subunits were docked with one another, even though the seatbelt latch and tensor disulfides could not be formed. It also revealed that the NH_2-termi-

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

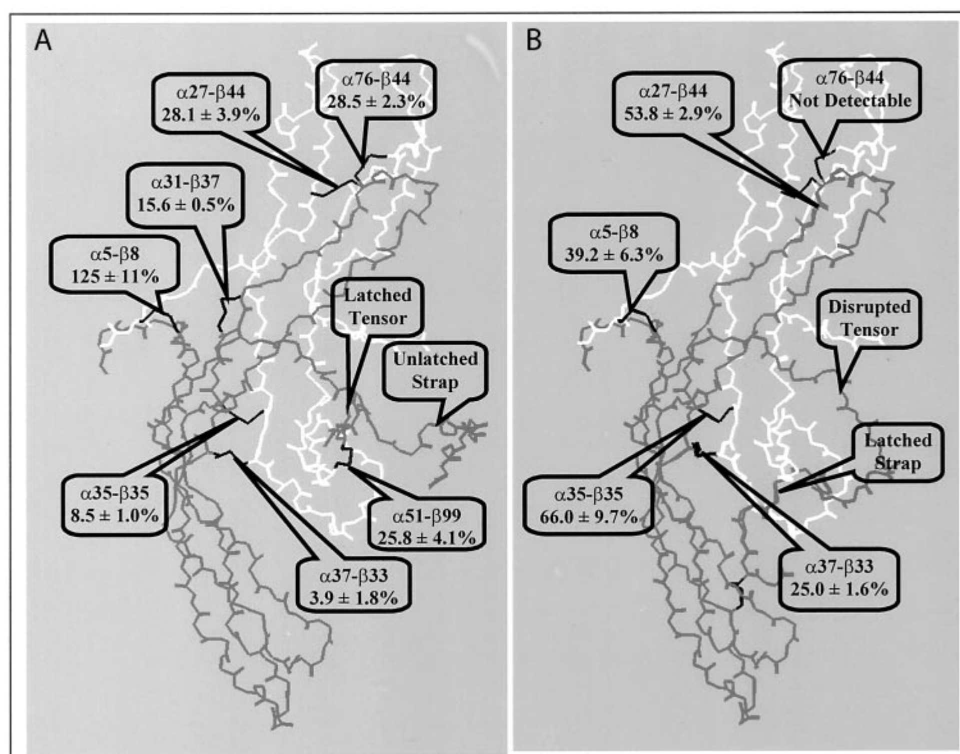

FIG. 2. **Contacts in the wraparound (*left panel*) and threading pathways (*right panel*).** Analogs of hCG β-subunit that lack the ability to form the seatbelt latch disulfide (β26-β110) do not combine with the α-subunit to form a stable heterodimer unless they contain an intersubunit disulfide cross-link. The disulfide bonds identified by the *callouts* in the *left panel* are located at sites that permit the formation of intersubunit disulfide bonds in hCG. The abilities of particular disulfides to stabilize the docked heterodimer are indicated on the figure as percentages that represent the amount of heterodimer formed when the seatbelt cannot be latched compared with the amount formed when the seatbelt can be latched. Elimination of the tensor disulfide (β93-β100) by replacing βCys^{93} and βCys^{100} with alanine also prevented the β-subunit from combining stably with the native α-subunit. Introduction of the disulfide bonds shown in the *callouts* in the *right panel* permitted formation of the amounts of heterodimer indicated in the callouts relative to that in analogs that can form the tensor disulfide. We assume that the amount of stable heterodimer formed is proportional to the time that the cysteines involved in the cross-link are adjacent to one another in the docked complex. The *white* and *gray lines* represent the backbones of the α- and β-subunits. The *black lines* depict the indicated disulfides. The position of the seatbelt strap and seatbelt are indicated. The positions of loop α2 and the seatbelt are assumed to be disordered. The remainder of the structure is similar to that of hCG. Although each figure depicts several disulfides for purpose of comparison, only one disulfide was studied in a given experiment. Values are average ± S.E. of at least 3 studies and in some cases, many more.

nal portions of both types of β-subunits appear to contact the NH_2-terminal portions of the α-subunit during subunit docking. The relative differences in the amounts of cross-linked docked subunits observed are impossible to interpret, however. They might indicate that contacts between the NH_2-terminal ends of the subunits are favored more when the seatbelt is unlatched than when the tensor disulfide is disrupted. Then again, they might also reflect the tendency of seatbelt residue βCys^{110} to form a disulfide with βCys^{8} in hCGβ-R8C,C93A,C100A (18), which would have rendered βCys^{8} incapable of forming an intersubunit disulfide with αCys^{5}. Consequently, only that fraction of hCGβ-R8C,C93A,C100A in which the seatbelt is latched to βCys^{26} would be capable of being cross-linked by a disulfide.

Parts of loop β2 contact loops α1 and α3 in hCG (1, 2) and in hFSH (3). To learn if these portions of the hCG β-subunit might participate in docking, we tested the abilities of cysteines that had been introduced in place of loop α1 residue αQ27C and loop α3 residue αV76C to form intersubunit disulfides with β-subunit analogs that contain a cysteine in place of hCG loop β2 residue βV44C. Co-expression of α-Q27C or α-V76C with hCGβ-V44C led to the formation of cross-linked heterodimers, all of which were acid stable. The α27-β44 and α76-β44 disulfides each stabilized 28% of the complexes containing the β-subunit that cannot latch its seatbelt. They were not as effective as the disulfide in the subunit NH_2 termini (Fig. 2, *left*). This suggested that when the hCG seatbelt is unlatched, the subunits dock in orientations that favor the formation of NH_2-terminal contacts relative to those between loop β2 and loops α1 and α3.

The α27-β44 disulfide also rescued heterodimers that are unable to form the tensor disulfide. As a result, co-expression of α-Q27C and hCGβ-V44C,C93A,C100A led to 54% as much heterodimer that was formed when α-Q27C was expressed with hCGβ-V44C (Fig. 2, *right*). This was as good or better than the α5-β8 disulfide (*i.e.* 39%). Remarkably, the α76-β44 disulfide between αV76C in loop α3 and βV44C in loop β2 did not rescue docked complexes containing hCGβ-V44C,C93A,C100A (Fig. 2, *right*), an observation that remains puzzling. We did not expect to find that the α27-β44 disulfide would rescue docked complexes containing β-subunits that cannot form their tensor disulfides better than those that cannot latch their seatbelts. This was because hCGβ-V44C,C93A,C100A has the potential to latch its seatbelt to βCys^{44} and, as a consequence, this cysteine would not be capable of being cross-linked to residue αCys^{27} in α-Q27C. In contrast, it is not possible for hCGβ-C26A,V44C,C110A to latch its seatbelt to βCys^{44}. Thus, the lower ability of the α27-β44 disulfide to rescue docked complexes containing an unlatched seatbelt may indicate that before it is latched, the seatbelt may impede the formation of contacts between the loops α1 and α3 with loop β2.

We studied potential contacts between loop α2 and either the cystine knot or loop β1 using analogs that can form intersubunit disulfide bonds between residues α35-β35 and α37-β33.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

Both disulfides have been shown to cross-link the subunits in hCG (7). Each rescued docked complexes containing β-subunits having latched seatbelts and disrupted tensor disulfides better than complexes containing β-subunits with unlatched seatbelts and intact tensor disulfides. Thus, the α35-β35 disulfide rescued 66% of the heterodimer containing α-R35C and hCGβ-A35C,C93A,C100A (Fig. 2, *right*), but only 8.5% of the heterodimer containing α-R35C and hCGβ-C26A,A35C,C110A (Fig. 2, *left*). The α37-β33 disulfide rescued 25% of the heterodimer containing α-Y37C and hCGβ-I33C,C93A,C100A (Fig. 2, *right*), but only 3.9% of the heterodimer containing α-Y37C and hCGβ-C26A,I33C,C110A (Fig. 2, *left*). The observations that the α35-β35 and α37-β33 disulfides formed more readily in analogs of hCGβ-C93A,C100A, which have latched seatbelts and disrupted tensor disulfides (Fig. 2, *right*), than in analogs of hCGβ-C26A,C110A, which have unlatched seatbelts and intact tensor disulfides (Fig. 2, *left*), suggested that loop α2 residues αArg^{35} and αTyr^{37} are more highly constrained during threading than wrapping. This would lead to increased contacts between the subunits, which would be expected to increase the stability of the docked complex.

Efforts to detect other potential contacts that might stabilize the docked complex prior to assembly by the wraparound pathway led us to test the ability of the α31-β37 disulfide to secure the heterodimer. This disulfide was found to stabilize an hCG analog formed by co-expressing hCGβ-Y37C with α-C7A (8) and was observed to rescue 15.6% of this material when hCGβ-C26A,Y37C,C110A was co-expressed with α-C7S (Fig. 2, *right*). We did not repeat these studies with analogs that are unable to form the tensor loop, because disulfides on either side of α31-β37 (*i.e.* α27-β44, α35-β35, and α37-β33) had already been found to rescue a much larger fraction of the heterodimer (Fig. 2, *right*). Considered together, these studies indicated that contacts near the cystine knots were likely to make a greater contribution to the threading pathway than to the wraparound pathway. The finding that these areas of the subunits are less likely to contact one another when the seatbelt is unlatched may reflect the ability of the unlatched seatbelt to disrupt contacts between the subunits, a topic to be considered later.

We anticipated that the seatbelt would make extensive contacts with residues in loop α2 during the threading pathway and that we would not be able to distinguish these from contacts made during docking. Indeed, we had already found that disulfide bonds appear to form between unpaired cysteines in loop α2 and the tensor disulfides during threading (18). To identify contacts between the seatbelt and the α-subunit that might facilitate docking in the wraparound pathway, we took advantage of a disulfide that forms between the seatbelt and loop α2, *i.e.* α51-β99 (7, 8). This disulfide rescued heterodimers containing α-K51C with hCGβ-C26A,D99C,C110A to 25.8% of the level observed when α-K51C was co-expressed with hCGβ-D99C (Fig. 2, *left*). This suggested that the tensor loop can participate in contacts with loop α2 while the seatbelt is unlatched. As noted later, we anticipate that hydrogen bonds between the backbone atoms of loop α2 residues αVal^{53}-αGlu^{56} and seatbelt residues βThr^{98}-βGly^{101}, which include part of the tensor loop, are necessary for efficient completion of assembly by the wraparound pathway. These contacts do not appear to stabilize NH_2-terminal portions of loop α2 that contact the β-subunit cystine knot, however, because analogs that are unable to latch their seatbelts were not rescued effectively by the α31-β37, α35-β35, or α37-β33 disulfides (Fig. 2, *left*).

The abilities of intersubunit disulfides to rescue docked complexes containing β-subunits that cannot latch their seatbelts or form the tensor disulfide suggest that the manner in which subunits dock is similar but nonidentical during assembly by threading and wrapping mechanisms. The finding that the α5-β8 disulfide rescued the formation of both types of heterodimers supported the notion that contacts in the NH_2-terminal region are important for the assembly of hCG and other lutropins. This is consistent with the observation that deletion of residues in the NH_2 terminus of the β-subunit reduced secretion of hCG analogs in which the seatbelt is latched normally (10, 11).

TABLE I
Influence of NH_2 terminal hCGβ residues 1–7 and 2–8 on heterodimer assembly by threading and wraparound pathways

COS-7 cells were transfected with the indicated constructs in triplicate and heterodimer secreted into the medium was quantified in A113/^{125}I-B110 sandwich immunoassays.

Data row	α-Subunit	β-Subunit	Total heterodimer
			ng/50 μll ± S.E.
Study 1, seatbelt latched to β26 or α37			
1	Native	hCGβ	14.73 ± 0.05
2	Native	δ1,7-hCGβ	1.30 ± 0.02
3	α-Y37C	hCGβ-C26A	5.92 ± 0.23
4	α-Y37C	δ1,7-hCGβ-C26A	<0.1
Study 2, seatbelt latched to β26 or α43			
5	Native	hCGβ	27.75 ± 2.93
6	Native	δ1,7-hCGβ	1.64 ± 0.09
7	Native	δ2,8-hCGβ	<0.1
8	α-S43C	hCGβ-C26A	7.56 ± 0.10
9	α-S43C	δ1,7-hCGβ-C26A	2.04 ± 0.13

The ability of the α5-β8 disulfide to stabilize heterodimers lacking the abilities to latch their seatbelts suggested that contacts between the NH_2-terminal portions of the subunits may have a dominant role during assembly by the wraparound pathway. To test this possibility, we monitored the abilities of analogs to form cross-linked heterodimers during assembly that can occur only by the wraparound pathway (Table I). Elimination of residues 1–7 or 2–8 reduced heterodimer secretion substantially (Table I). They also reduced assembly of heterodimers in which the seatbelt is latched to either α-subunit residue 37 (Table I, study 1) or to α-subunit residue 43 (Table I, study 2). This suggests that NH_2-terminal contacts are likely to have a role in subunit docking even though they are not essential for docking.

The contribution of NH_2-terminal contacts to assembly may explain our inability to detect assembly of hTSH and hFSH by a wraparound mechanism. These β-subunits have only one and two residues in their NH_2-terminal ends, respectively. Therefore, the finding that hTSH and hFSH were unable to form heterodimers by a wraparound mechanism (17) may indicate that contacts between the NH_2-terminal portions of both subunits are more important for assembly by a wrapping mechanism than for assembly by threading.

Docking Is Readily Reversible, a Phenomenon That May Delay Most hCG Assembly Until the Seatbelt Is Latched—Experiments described next were performed during efforts to identify rate-limiting steps in glycoprotein hormone assembly and thereby learn why most hCG is assembled by a threading mechanism. These studies depended on our abilities to identify the relative rates of subunit docking, threading, and wrapping in the ER. We monitored these processes using the α5-β8 disulfide because of its ability to trap and rescue docked complexes in which the seatbelt is unlatched, a phenomenon that was required to detect early stages in the wraparound pathway. During these studies we compared the abilities of hCGβ to compete with hCGβ-R8C and hCGβ-R8C,C26A,C110A for α-Q5C for heterodimer formation. We reasoned that if threading or wrapping were not rate-limiting and occurred immediately after the subunits dock, then hCGβ would compete effi-

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE II
Competition of various hCG β-subunit analogs for α-O5C

All β-subunit analogs shown were co-transfected into COS-7 cells with α-Q5C. Measurements reflect the results of sandwich immunoassays employing A113/^{125}I-B110 (total heterodimer and acid stability) or A113/^{125}I-B111. Data in the third column represent the ratio of measurements in the B111/B110 assays multiplied by 100.

Data row	β-Subunit(s)	Total heterodimer	Acid stability	B111 positive dimer
		ng/50 μl ± S.E.	*% total ± S.E.*	
1	hCGβ-R8C	4.59 ± 0.38	104.5 ± 2.6	113.8 ± 1.1
2	hCGβ-R8C + hCGβ	5.76 ± 0.32	87.6 ± 4.9	112.1 ± 1.9
3	hCGβ-R8C,C26A,C110A	8.91 ± 1.62	97.4 ± 2.9	67.6 ± 3.0
4	hCGβ-R8C,C26A,C110A + hCGβ	8.25 ± 0.90	97.6 ± 4.9	72.0 ± 0.8
5	hCGβ-R8C,C26A	7.82 ± 1.45	102.4 ± 7.2	12.4 ± 0.5
6	hCGβ-R8C + hCGβ-R8C,C26A	4.75 ± 0.33	85.4 ± 3.2	90.2 ± 4.4
7	hCGβ-R8C,C26A,C110A + hCGβ-R8C,C26A	6.94 ± 0.30	105.7 ± 3.7	46.9 ± 1.9

ciently with hCGβ-R8C or hCGβ-R8C,C26A,C110A for heterodimer formation. Consequently, a substantial fraction of the heterodimer formed in the presence of hCGβ would lack an intersubunit disulfide and dissociate at pH 2, 37 °C. In contrast, if the subunits docked and undocked faster than the heterodimer became stabilized by threading or wrapping mechanisms, then hCGβ-R8C and hCGβ-R8C,C26A,C110A would constitute most of the β-subunit in the heterodimer. This is because formation of the α5-β8 disulfide cross-link, a reaction unlikely to be reversed quickly, would permit heterodimers containing these β-subunit analogs to accumulate at the expense of heterodimers lacking the ability to form this disulfide.

To determine the relative rates of docking, undocking, and assembly, we compared the abilities of hCGβ to inhibit the formation of acid-stable heterodimers containing α-Q5C and hCGβ-R8C or hCGβ-R8C,C26A,C110A. Preliminary studies showed that heterodimers containing hCGβ and α-Q5C are acid labile, making them readily distinguished from those that contained α-Q5C and hCGβ-R8C or α-Q5C and hCGβ-R8C,C26A,C110A. For example, 3 days following the co-transfection of COS-7 cells with hCGβ and α-Q5C, we observed that only 1.9 ± 0.6% of the total heterodimer in the medium (*i.e.* 13.6 ± 1.15 ng/50 μl) survived treatment at pH 2 for 30 min at 37 °C. In contrast, all the heterodimer formed by co-transfecting α-Q5C and hCGβ-R8C was stable after this treatment (Table II, data row 1), showing that it was cross-linked by an intersubunit disulfide, as expected. Most of the heterodimer formed following co-transfection of α-Q5C with a mixture of hCGβ-R8C and hCGβ was acid stable, indicating that hCGβ-R8C out competed hCGβ for assembly of heterodimers with α-Q5C (Table II, data row 2). We usually observed that 85% or more of the heterodimer was acid stable when equal amounts of hCGβ and hCGβ-R8C were used during transfection and, in some experiments, we did not detect any competition of hCGβ.

We observed also that hCGβ competed poorly with hCGβ-R8C,C26A,C110A, an analog that cannot latch its seatbelt (Table II, data rows 3 and 4). In most studies, heterodimers formed when α-Q5C was co-transfected with mixtures of hCGβ and hCGβ-R8C,C26A,C110A were at least 90% as stable as those that had been formed when α-Q5C was co-transfected only with hCGβ-R8C,C26A,C110A. This suggested that they contained only small amounts of hCGβ, a finding that was confirmed by their abilities to be recognized by antibody B111. B111 is an antibody that recognizes a site on the hCG β-subunit that includes β-subunit residues near βCys^{26} and βCys^{110}. This antibody can recognize single chain hCG analogs in which both of these latched cysteines are replaced by alanine, albeit not as well as hCG (5). It does not recognize analogs in which the seatbelt is latched to the α-subunit (5) or those in which residues near βCys^{110} are derived from hLH, hFSH, or hTSH. The ability of hCGβ-R8C,C26A,C110A to out compete hCGβ shows that docking occurs efficiently before the seatbelt is latched and that docked complexes containing unlatched or latched β-subunits are likely to dissociate before being assembled into stable heterodimers by a wraparound mechanism.

The ability of hCGβ-R8C,C26A,C110A to out compete hCGβ indicated that formation of the α5-β8 disulfide occurs rapidly when the seatbelt is unlatched. Thus, it is conceivable that the ability of hCGβ-R8C to out compete hCGβ might reflect its ability to dock with α-Q5C before its seatbelt is latched. To test this possibility, we monitored the ability of hCGβ-R8C to compete with hCGβ-R8C,C26A for heterodimer formation with α-Q5C. We used hCGβ-R8C,C26A, an analog that cannot latch its seatbelt, in place of hCGβ-R8C,C26A,C110A in these studies because it combined with α-Q5C to form heterodimers that were not recognized as well by antibody B111 (Table II, data rows 3 and 5), a phenomenon that increased the sensitivity of this assay. Heterodimers formed when α-Q5C was co-expressed with a mixture of hCGβ-R8C and hCGβ-R8C,C26A contained more hCGβ-R8C than hCGβ-R8C,C26A (Table II, data row 6). The dominance of hCGβ-R8C in this assay suggested that analogs in which the seatbelt is latched dock better than those in which the seatbelt is unlatched. Control studies showed that hCGβ-R8C,C26A competed effectively with hCGβ-R8C,C26A, C110A, a related analog that also lacks the ability to latch its seatbelt (Table II, data row 7). Considered together, these findings suggested that hCGβ-R8C and hCGβ are likely to compete for α-Q5C after their seatbelts are latched. Thus, the ability of hCGβ-R8C to out compete hCGβ suggests that a significant fraction of hCGβ dissociates from the α-subunit before it can be incorporated into heterodimers by a threading mechanism. The finding that docking is reversible whether the seatbelt is latched or not suggests that threading and wrapping are rate-limiting steps in assembly. Repeated dissociation of the docked complex would provide additional time for the seatbelt to become latched, a phenomenon that would cause most assembly to take place by a threading mechanism as was found (17).

Whereas the Seatbelt Is Not Essential for Docking, It Can Interfere with Docking When It Is Unlatched—To learn if the seatbelt is essential for docking, we tested the abilities of hCGβ-R8C,δ92,C26A, hCGβR8C,δ101, hCGβ-R8C,δ93:100D, and hCGβ-R8C,δ111 to form cross-linked heterodimers when they were co-expressed with α-Q5C. The first of these β-subunit analogs lacks the tensor loop, the seatbelt strap, and the COOH terminus. The second analog lacks the seatbelt strap and COOH terminus, and the third analog lacks the tensor loop. All three analogs form cross-linked heterodimers, albeit not as well as hCGβ-R8C,δ111, a β-subunit analog that lacks the hCG β-subunit COOH terminus (Table III). These data showed that the seatbelt is not essential for docking even though it may enhance docking, affect formation of the α5-β8 disulfide, or promote the secretion of the cross-linked complex. They also showed that the carboxyl-terminal end of the seatbelt

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE III
Influence of the seatbelt and Coottterminus on the assembly of cross-linked heterodimers

The β-subunit was truncated at the indicated residue. The amounts of cross-linked heterodimer that formed were normalized relative to the amount of heterodimer containing hCGβ-R8C expressed as 100%, which enabled comparisons of multiple studies. The average amount of heterodimer containing hCGβ-R8C was 4.73 ± 0.93 ng/50 μl (four independent studies).

Row	Construct used in transfection	Percent ± S.E.
1	α-Q5C + hCGβ-R8C	100.0 ± 3.3
2	α-Q5C + hCGβ-R8C,δ111	108.3 ± 7.6
3	α-Q5C + hCGβ-R8C,δ101	9.8 ± 0.4
4	α-Q5C + hCGβ-R8C,C26A,δ92	22.9 ± 1.8
5	α-Q5C + hCGβ-R8C,δ 93:100D	4.4 ± 0.9

TABLE IV
Many β-subunit analogs capable of latching their seatbelts to an intrasubunit cysteine usually inhibited hCG assembly better than those that could not do so, even though both forms are unable to complete the assembly process

The indicated β-subunit was co-transfected into COS-7 cells with the native α- and hCG β-subunits. Heterodimer formation was monitored in A113/^{125}I-B110 sandwich immunoassays.

α-Subunit + hCGβ	β-Subunit Analog Inhibitor	Seatbelt Status	Heterodimer Total/50μl (ng ± SEM)
Native	None	Latched	2.78 ± 0.42
	hCGβ-C26A	Unlatched	3.00 ± 0.49
	hCGβ-L5C,C26A	Latched	1.72 ± 0.20
	hCGβ-R6C,C26A	Latched	1.43 ± 0.06
	hCGβ-R8C,C26A	Latched	1.35 ± 0.11
	hCGβ-E19C,C26A	Latched	1.79 ± 0.10
	hCGβ-A35C,C26A	Latched	2.25 ± 0.02
	hCGβ-Y37C,C26A	Latched	0.65 ± 0.09
	hCGβ-F64C,C26A	Latched	1.96 ± 0.14
	hCGβ-N77C,C26A	Latched	2.16 ± 0.02
	hCGβ-A83C,C26A	Latched	1.79 ± 0.06

is not essential for docking, a phenomenon that is consistent with the finding that this portion of the β-subunit is not required for efficient heterodimer secretion (12).

The finding that the seatbelt may contribute to docking did not resolve a key goal of these studies, namely to learn why most hCG is assembled by a threading pathway. To learn if the seatbelt might interfere with docking or destabilize the docked complex when it is unlatched, we tested the abilities of β-subunit analogs to disrupt hCG assembly when their seatbelts were unlatched or latched to non-native sites. We anticipated that movements of the unlatched seatbelt might offset contributions made by interactions of the tensor loop with α-subunit loop 2 that enabled formation of the α51-β99 disulfide (Fig. 2, *left*). By altering the position of the seatbelt latch site, we anticipated that we would reduce the inhibitory influence of the seatbelt caused by its mobility. We assumed that if the ability of the unlatched seatbelt to inhibit docking was greater than its contribution to docking, these analogs would dock with the α-subunit better than the parental analog in which the seatbelt is unlatched, *i.e.* hCGβ-C26A. Because these analogs cannot be incorporated into the heterodimer, we expected that their relative abilities to dock with the α-subunit could be estimated by their abilities to inhibit hCG secretion. We observed that several β-subunit analogs in which the seatbelt was latched to alternate sites inhibited hCG assembly by 40% or more (Table IV). In contrast, hCGβ-C26A, the β-subunit analog lacking the ability to latch its seatbelt, did not inhibit assembly (Table IV, data rows 1 and 2). This supported the notion that analogs having an unlatched seatbelt had reduced abilities to dock with the α-subunit, making them less able to inhibit the interactions between the native hCG α- and β-subunits. The finding that latching the seatbelt to several sites on the β-subunit appears to facilitate subunit docking shows that the ability of the unlatched seatbelt to disrupt subunit docking outweighs its positive contributions to subunit interactions. Combined with the finding that docking is reversible, which would provide additional time for the seatbelt to become latched, the finding that the unlatched seatbelt can interfere with docking or destabilize the docked complex would readily account for the dominance of the threading mechanism relative to the wraparound mechanism during hCG assembly (17).

Contacts between the Tensor Loop and α-Subunit Loop 2 Appear to Contribute to Latching during the Wraparound Pathway—We have observed that formation of the tensor disulfide appears to facilitate formation of the seatbelt latch disulfide during the folding of the free hCG β-subunit (18). The finding that residues of the tensor loop became cross-linked to loop α2 when α-K51C was expressed with hCGβ-C26A,D99C,C110A suggested that these portions of the α- and β-subunits interact. Analysis of hydrogen bonds in the hCG and hFSH heterodimers showed that several are formed between residues in loop α2 and the tensor loop (Fig. 3, *rightmost panel*). We anticipated that formation of these bonds and/or other contacts in this region may facilitate latching. To test this possibility, we compared the ability of B111 to recognize hCG analogs that lack the abilities to latch their seatbelts. As noted earlier, this antibody recognizes a conformation of the end of the seatbelt when it is latched to βCys[26] but not to other cysteines in either the α- or β-subunits (5, 17, 18). B111 can also recognize hCG analogs in which the seatbelt has a conformation similar to that in the heterodimer even when the position of the end of the seatbelt is not stabilized by the β26-β110 seatbelt latch disulfide. For example, we found that B111 can recognize single chain hCG analogs in which βCys[26] and βCys[110] are converted to alanine ~70% as well as hCG (5). We have also found that B111 can recognize heterodimers that are stabilized by an NH_2-terminal disulfide (*i.e.* between α5-β8) such as those that contain α-Q5C and hCGβ-R8C,C26A,C110A (Table V, data row 2). We anticipated that if the contacts between the tensor loop and α-subunit loop 2 contributed to the stability of the seatbelt, elimination of the tensor disulfide would adversely affect the ability of B111 to recognize hCG analogs lacking the abilities to latch their seatbelts. Control studies showed that elimination of the tensor disulfide by itself did not affect B111 binding (Table V, data row 3). Removal of both the tensor disulfide and the seatbelt latch disulfide reduced the ability of B111 to recognize the heterodimer (Table II, data row 4). This can be seen by comparing the ability of heterodimers containing hCGβ-R8C,C26A,C110A and those containing hCGβ-R8C,C26A,C93A,C100A,C110A to be recognized by B111 (Table V, data rows 2 and 4). These studies suggest that interactions between the seatbelt and the α-subunit that occur before the seatbelt is latched contribute to the ability of the seatbelt to find its latch site during assembly that occurs by a wraparound mechanism. This would also explain the reduction in heterodimer formation that occurs when hCG analogs lacking the ability to form the tensor disulfide are forced to latch their seatbelts to a cysteine in the α-subunit (Table V, data rows 5–7).

DISCUSSION

Subunit Docking in the ER—These studies were initiated to identify regions of the hCG subunits most likely to contact one another during the wrapping and threading pathways and to use this information to learn why most hCG is assembled by a threading mechanism. We identified contact regions using

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

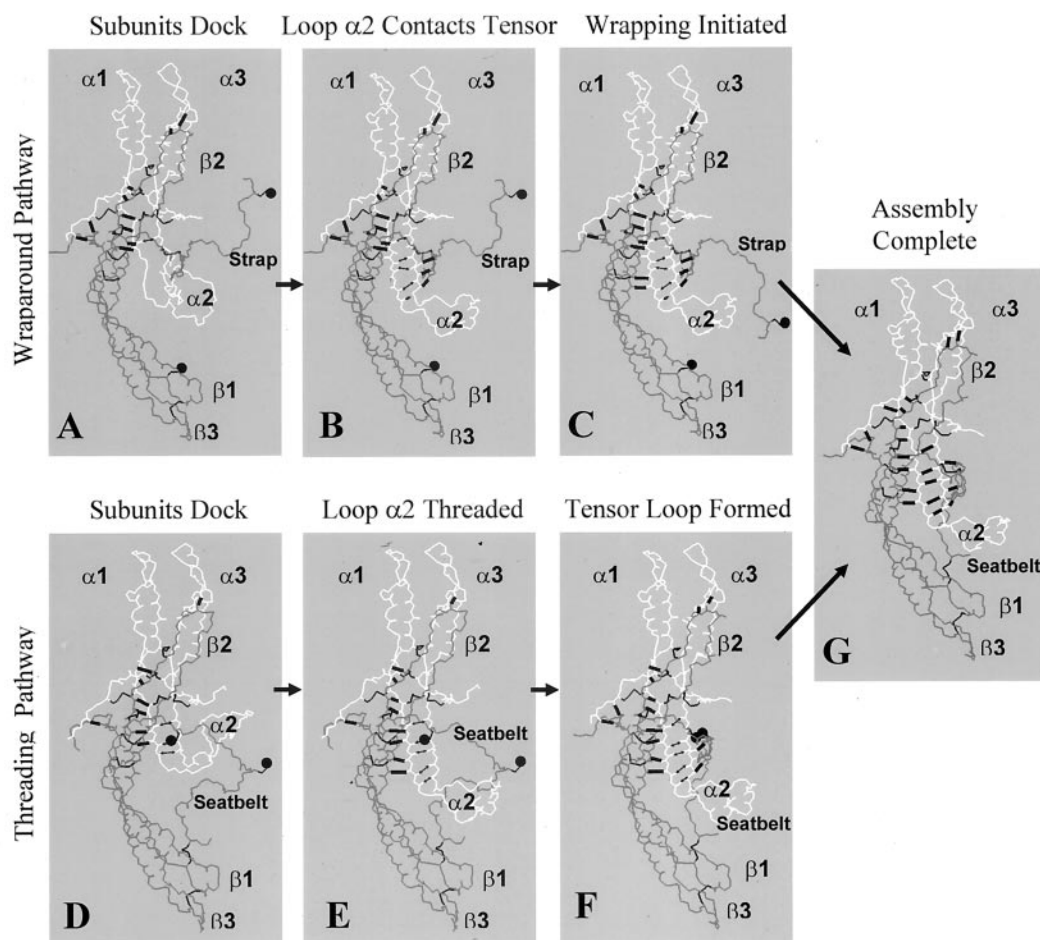

FIG. 3. **Models of assembly in the wraparound pathway (*upper panels*) and threading pathway (*lower panels*).** During subunit docking in both pathways contacts are formed between residues in the NH_2-terminal ends of both subunits and between NH_2-terminal residues in β-subunit loop β2 and α-subunit loop α1 (*panels A* and *D*). Other contacts are influenced by the seatbelt. When the seatbelt is unlatched, residues in loop β2 are more likely to contact loop α3 than when the seatbelt is latched. When the seatbelt is latched, residues within and adjacent to the cysteine knots are more likely to contact one another. Assembly of the heterodimer requires that loop α2 form an anti-parallel β-sheet (*panels B* and *E*). The tensor disulfide appears to remain intact during the wraparound pathway (*panels A–C*), a phenomenon that facilitates the formation of hydrogen bonds between the tensor loop and loop α2 (*panel B*). This position favors the formation of hydrogen bonds within loop α2 and between loop α2 and the β-subunit cystine knot. As a result, the strap region of the seatbelt appears to become stabilized in a position that facilitates latching the end of the seatbelt strap to loop β1 in most vertebrate hormones (*panel C*) or to the NH_2 terminus of the β-subunit in salmon and other teleost fish follitropins. This would explain the ability of the tensor disulfide to facilitate assembly by the wraparound pathway and to enhance B111 binding to analogs that cannot latch their seatbelts. In contrast, the seatbelt is latched during assembly by the threading pathway (*panels D–F*), a phenomenon that requires loop α2 to be threaded beneath the seatbelt. Threading occurs while the tensor disulfide is disrupted and appears to be driven by formation of hydrogen bonds between the β-subunit cystine knot and loop α2 (*panel E*), a process that would effectively "pull" loop α2 and its attached oligosaccharide beneath the seatbelt. Formation of these hydrogen bonds explains the abilities of the α35-β35 and α37-β33 intersubunit disulfides to stabilize heterodimers that cannot form the tensor disulfide. Formation of hydrogen bonds between loop α2 and portions of the tensor loop stabilize βCys^{100} near βCys^{93} (*panel F*), a phenomenon that facilitates reformation of the tensor disulfide and accounts for its greater stability in the heterodimer. Completion of either pathway yields a heterodimer in which the anti-parallel β-sheet portion of loop α2 is sandwiched between two portions of the β-subunit by hydrogen bonds (*panel G*). The importance of these hydrogen bonds in stabilizing the heterodimer is supported by the ability of low pH or urea to dissociate the heterodimer while the seatbelt remains latched. Movements of loop α2 that would tend to destabilize the heterodimer are restricted by the strap region of the seatbelt when it is latched. The dominance of the threading pathway during the assembly of hCG appears because of the ability of the unlatched seatbelt to destabilize the docked subunit complex. α-Subunit residues, *white*; β-subunit residues, *dark gray*; intersubunit H-bonds prior to assembly, *solid bars*; loop α2 H-bonds prior to completion of assembly, *thin arrows* containing *two heads*; thiol atoms in βCys^{26} and βCys^{110}, *black spheres* in *top panels*; thiol atoms in βCys^{93} and βCys^{100}, *black spheres in bottom panels*.

disulfide cross-links to trap unstable docked complexes that contained β-subunit analogs that cannot latch their seatbelts or that cannot form the tensor disulfide. These analogs should mimic the structures of the β-subunit at the time assembly is occurring by wrapping and threading mechanisms, respectively.

We considered two technical difficulties before beginning these studies, the first of which involved the selection of disulfide cross-links. We used only those cross-links that can form in the native heterodimer. While this may have caused us to miss early transient subunit interactions, it should have enabled us to detect contacts that form between the time the subunits dock productively and the time that wrapping and threading are nearly complete. Second, we expected this approach to work best with β-subunit analogs in which βCys^{110} was replaced with alanine. The mobility of the hCG seatbelt prior to docking enables βCys^{110} to become latched to cysteines that have been added to the β-subunit rather than βCys^{26}, its normal site (18). Based on our earlier findings (Ref. 18, Table I), we were particularly concerned that this might affect β-subunit constructs containing βI33C and βA35C, residues needed to observe contacts with loop α2. Because βCys^{110} was present only in constructs that were used to monitor threading, this problem would have affected our ability to monitor the threading pathway, not the wraparound pathway. Fortunately, we were able

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

The Journal of Biological Chemistry

TABLE V
The tensor disulfide can influence the location of the seatbelt following assembly by the wraparound pathway and facilitate latching to cysteines in the α-subunit

The total amount of heterodimer was monitored in A113/^{125}I-B110 assays, which do not depend on the position of the seatbelt. The position of the seatbelt was monitored in A113/^{125}I-B111 assays, which depend on the ability of seatbelt residue 110 to be adjacent to loop β1 residue 26. Normally, these two residues are held adjacent by the seatbelt latch disulfide. As seen by comparing the B111 assay results in data rows 2 and 3, the ability of the unlatched seatbelt to occupy this position depends on formation of the tensor disulfide (*i.e.,* that formed by residues βCys^{93} and βCys^{100}. Formation of the tensor disulfide was also essential for optimum cross-linking of the seatbelt to a cysteine added to the α-subunit, as seen by the reduced amount of heterodimer formed in data row 6 relative to data row 5.

Data row	α- and β-subunit analogs	Total heterodimer	B111 assay
		ng/50 μl ± S.E.	*% total ± S.E.*
1	α-Q5C + hCGβ-R8C	8.01 ± 1.00	100.1 ± 4.1
2	α-Q5C + hCGβ-R8C,C26A,C110A	12.33 ± 1.71	57.7 ± 2.8
3	α-Q5C + hCGβ-R8C,C93A,C100A	3.86 ± 0.46	98.9 ± 4.1
4	α-Q5C + hCGβ-R8C,C26A,C93A,C100A,C110A	0.27 ± 0.02	14.0 ± 1.7
5	α-L41C + hCGβ	5.20 ± 0.84	Not tested
6	α-L41C + hCGβ-C26A	3.68 ± 0.58	Not tested
7	α-L41C + hCGβ-C26A,C93A,C100A	0.99 ± 0.03	Not tested

to detect these contacts readily despite the potential difficulty of doing so (Fig. 2, *right*).

Some areas of the subunits appeared to contact one another well in both assembly mechanisms. These include interactions between residues in the α- and β-subunit NH_2 termini and interactions between parts of loops α1/α3 and parts of loop β2 (Fig. 2, *left* and *right*). Other contact regions, such as those involving loop α2 and residues near the cystine knots, differ significantly in complexes that are thought to be precursors of threading and wrapping. For example, a larger portion of loop α2 appears to contact the β-subunit core during threading (*i.e.* when the seatbelt is latched and the tensor disulfide is disrupted) than during wrapping (*i.e.* when the tensor disulfide is formed and the seatbelt is unlatched). Consequently, intersubunit disulfides α35-β35 and α37-β33 rescued complexes containing β-subunits that cannot form the tensor disulfide much better than those that cannot latch their seatbelts (Fig. 2). This phenomenon may contribute to the dominance of the threading pathway during assembly and is particularly remarkable because these contacts were more likely to be underestimated in complexes containing β-subunits that cannot form the tensor disulfide.

Earlier reports that deletion of the NH_2-terminal portion of the hCG β-subunit disrupted heterodimer secretion (10, 11) indicated that intersubunit contacts involving this portion of the β-subunit might participate in docking. This is supported by the finding that β-subunits that cannot latch their seatbelts or form the tensor disulfide were stabilized efficiently by the α5-β8 intersubunit disulfide (Fig. 2). Because the follitropin and thyrotropin β-subunits lack an NH_2-terminal extension, contacts in these regions appear to be limited to lutropins. The requirement for these NH_2-terminal residues was lessened in analogs containing portions of the hFSH seatbelt (11). The inability of the hFSH and hTSH β-subunits to form these contacts may be responsible for our failure to detect any assembly of hFSH and hTSH by a wraparound mechanism (17).

Intersubunit disulfides between loops β2 and α1 rescued heterodimer formation in both pathways, suggesting that contacts between these regions also contribute to subunit docking during assembly by either mechanism (Fig. 2, *left* and *right*). Formation of contacts that involve loop β2 might appear surprising because this is one of the least conserved portions of the β-subunit and differs substantially in lutropins, follitropins, and thyrotropins (13). Contacts in these regions of the hCG heterodimer may be stabilized by hydrogen bonds between backbone atoms of residues αCys^{28}-βThr^{42}, αGly^{30}-βThr^{40}, and αCys^{32}-βCys^{38} (1, 2). These correspond to parts of the α-subunit cystine knot and the NH_2-terminal end of loop β2. Other hydrogen bonds are likely to involve backbone atoms of αSer^{34}-βGly^{36}, residues in the NH_2-terminal end of loop α2 and the β-subunit cystine knot. Thus, both cystine knots appear to contribute to docking, particularly when the tensor disulfide is disrupted. In addition, interactions between the hydrophobic side chains of loop β2 residues hCGβ-Val^{44} and hFSHβ-Val^{38} with a hydrophobic patch on a concave surface of loops α1 and α3 that includes human α-subunit residues αPhe^{17}, αPhe^{18}, αPhe^{74}, αVal^{76}, and possibly αIle^{25} and αVal^{70} appear to contribute to the stability of the docked subunits when the tensor disulfide is disrupted or the seatbelt is unlatched (Table VI). Sequence alignments show that hydrophobic residues are found at these positions in most vertebrate α-subunits and in most gonadotropin β-subunits. We anticipate that contacts in this region of the thyrotropin β-subunit involve hydrogen bonds between residues corresponding to hTSHβ-Asn^{37} and the α-subunit or hydrophobic interactions between the α-subunit and residues corresponding to hTSHβ-Leu^{40},Phe^{41},Leu^{42}. Thus, contacts between loops α1/α3 and loop β2 would be expected to contribute to the stability of the docked complex despite the differences in loop β2 found in lutropins, follitropins, and thyrotropins.

Loop α2 is disordered in the free α-subunit (14). Following heterodimer assembly, the portion of this loop near the α-subunit cystine knot forms two anti-parallel strands (1–3). These are stabilized by residues in the β-subunit cystine knot and by parts of the tensor loop (Fig. 3). The abilities of disulfides to stabilize intermediates that serve as prototypes for the wraparound and threading pathways (Fig. 2, *left* and *right*) suggest how loop α2 acquires this position. The α35-β35 and α37-β33 disulfides rescued analogs thought to model threading (Fig. 2, *right*) better than those thought to model wrapping (Fig. 2, *left*). This implies that residues near the NH_2-terminal end of loop α2 are nearer the β-subunit cystine knot during threading than wrapping. During the wrapping pathway (Fig. 2, *left*), the α51-β99 disulfide rescued heterodimer formation better than the α35-β35 and α37-β33 disulfides (Fig. 2, *left*). Thus, tensor loop residue βAsp^{99} is nearer loop α2 residue αLys^{51} during wrapping than cystine knot residue βAla^{35} is to αArg^{35} and βIle^{33} is to αTyr^{37}. These data suggest how assembly is driven in the threading and wraparound pathways. During threading, the β-sheet begins forming near the cystine knot and terminates with the formation of hydrogen bonds that stabilize loop α2 near tensor loop residue βCys^{100} (Fig. 3, *lower panels*). This constrains βCys^{100} near βCys^{93} and enables the tensor disulfide to reform, which completes assembly. This sequence of events is reversed in the wraparound pathway (Fig. 3, *upper panels*), which begins while the intact tensor disulfide is near αLys^{51} and terminates with the formation of hydrogen bonds between the NH_2-terminal end of loop α2 and the cystine knot.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

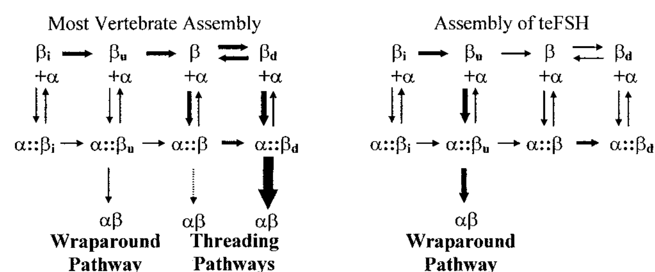

FIG. 4. **Summary of glycoprotein hormone assembly in the ER.** *Left diagram*, assembly of glycoprotein hormones in which the seatbelt is latched to loop $\beta 1$. As reported here, the β-subunit can dock with the α-subunit before it has folded completely. Docking is reversible, however, and only a few forms of the docked complex appear to be assembled into heterodimers in significant quantities. These include forms in which the tensor disulfide is latched and the seatbelt is unlatched (β_u) and those in which the tensor disulfide is disrupted and the seatbelt is latched (β_d). The subunits are also likely to dock while all the disulfides in the β-subunit are formed (β), but it remains to be determined if this docked complex can be assembled into a heterodimer in the ER unless the tensor disulfide can be unlatched while the subunits remain docked. As shown by the *broken arrow*, based on assembly that occurs *in vitro*, some heterodimers may assemble while the seatbelt is latched and the tensor disulfide is intact. The concentrations of subunits required for this type of assembly and the slow rate at which it occurs reduce the likelihood that it contributes significantly to assembly in the ER, however. The *widths* of the *solid arrows* indicate the most likely pathways for assembly of heterodimers in which the seatbelt is latched to loop $\beta 1$, which is the case for glycoprotein hormones in most vertebrates. *Right diagram*, assembly of glycoprotein hormones in which the seatbelt is latched to a cysteine in the β-subunit NH_2 terminus. teFSH is assembled by a wraparound route, most likely because the space between the latched seatbelt and the β-subunit core appears to be too small to accommodate efficient passage of loop $\alpha 2$, even when the tensor disulfide is disrupted. The ability of the seatbelt to be latched to the β-subunit prior to assembly would significantly inhibit assembly of these heterodimers. Other abbreviations: β_i, β-subunit intermediates before the seatbelt is latched and one or more of the remaining disulfides are disrupted; α::β, docked complexes containing the indicated β-subunits; $\alpha\beta$, assembled heterodimer.

Formation of the latter may require the seatbelt to be latched, albeit not necessarily to βCys^{26}, because the wraparound pathway can be used to assemble heterodimers in which the seatbelt is latched to cysteines in the α-subunit (5).

Docking Appears to Be Reversible Before or After the Seatbelt Is Latched, a Phenomenon That Would Favor Assembly by Threading—Previously, we found that the seatbelt is latched in most hCG β-subunit molecules before it is incorporated into the heterodimer by a threading mechanism (17). Efforts to determine how turnover of the docked complex affected assembly led us to monitor the abilities of β-subunit analogs to compete for the formation of heterodimers that were stabilized by the α5-β8 disulfide. These studies showed that the subunits dock readily before the hCG β-subunit seatbelt is latched. The poor ability of hCGβ to compete with hCGβ-R8C,C26A,C110A showed that formation of the α5-β8 disulfide occurred more rapidly than threading or wrapping, processes needed to stabilize heterodimers containing hCGβ and α-Q5C. The inability of the wraparound pathway to compete with the threading pathway for hCG assembly (17) shows that docked complexes having unlatched seatbelts are more likely to dissociate than complexes that have latched seatbelts. This provides additional time for seatbelts to become latched, a phenomenon that appears to be only slowly reversible at the redox potential of the ER and that would explain why the seatbelt is latched prior to most assembly (17). This would also reduce the amount of unlatched β-subunit substrate available for assembly by the wraparound pathway.

The likelihood that the docked complex will dissociate before wrapping is completed appears to be a significant problem for the assembly of heterodimers such as teFSH, which can be assembled only by a wraparound mechanism (19). The composition of the salmon FSH seatbelt appeared to inhibit threading when it was latched to loop $\beta 1$. Thus, the reversibility of docking would promote latching the seatbelt to loop $\beta 1$, which would impede assembly substantially (19). The need to reduce premature seatbelt latching may have caused the latch site to migrate to the NH_2 terminus of the teFSH β-subunit. In this site it might be latched better after the subunits dock because of interaction between loop $\alpha 2$ and the tensor loop that would constrain the end of the seatbelt near the latch site (Fig. 3, *upper panels*).

The Unlatched Seatbelt Can Interfere with Docking and/or Destabilize the Docked Complex—The ability of hCGβ-R8C to out compete hCGβ-R8C,C26A for heterodimer formation with α-Q5C indicated that the unlatched seatbelt may inhibit docking or destabilize the docked complex. We tested these possibilities by monitoring the abilities of β-subunit analogs to inhibit hCG assembly when the seatbelt was latched to alternate sites (Table IV). Most of these analogs inhibited hCG assembly more effectively than hCGβ-C26A, an analog that cannot latch its seatbelt. This finding suggests that the unlatched seatbelt interferes with docking and/or reduces the stability of the docked complex.

Analogs of hCGβ that can latch their seatbelts normally but that contain altered tensor loops were also effective inhibitors of hCG assembly (18). Whereas this might indicate that the tensor loop makes few contributions to docking, differences in the abilities of truncated hCGβ-R8C analogs to be cross-linked to α-Q5C suggest otherwise. Analogs that were truncated at residues 92, 101, or 111, lack the β-subunit COOH terminus. The first two of these also lack most of the seatbelt and the seatbelt strap region, respectively. While removal of the COOH terminus did not alter formation of the cross-linked heterodimer, elimination of the seatbelt and the strap region reduced heterodimer formation substantially (Table III). This showed that the seatbelt can contribute to docking, possibly through contacts of its tensor loop with loop $\alpha 2$ (Fig. 2, *left*).

What Drives Threading and Wrapping, Processes Needed to Complete Assembly?—The assembly of glycoprotein hormone heterodimers by threading and wrapping mechanisms may be driven by the formation of intrasubunit and intersubunit hydrogen bonds that involve parts of loop $\alpha 2$, the β-subunit cystine knot, and the tensor loop. This can be visualized by combining information of the apparent positions of the hormone subunits in the docked complexes (Fig. 2, *left* and *right*) with those in the crystal structures of hCG and hFSH. Following docking, assembly results in the formation of intersubunit hydrogen bonds between several residues in loop $\alpha 2$ and parts of the β-subunit cystine knot, intrasubunit hydrogen bonds between several residues within the anti-parallel strands of loop $\alpha 2$ and its tip, and intersubunit hydrogen bonds between parts of loop $\alpha 2$ and the seatbelt, notably the tensor region (Fig. 3). The sequential formation of these hydrogen bonds is likely to differ in the threading and wrapping pathways. In both cases, however, we suggest they form in "zipper-like" fashions to position loop $\alpha 2$ between the β-subunit cystine knot and the tensor loop where it can be stabilized readily by reformation of the tensor disulfide following threading or by latching the seatbelt following wrapping (Fig. 3).

Following threading, the position of tensor cysteine hCGβ-Cys^{100} would be stabilized at a site near hCGβ-Cys^{93}. This would facilitate reformation of the tensor disulfide and explain the increased stability of this disulfide in the heterodimer (Fig. 3). By reducing the size of the space beneath the seatbelt in the

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

The Journal of Biological Chemistry

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE VI

Conserved residues in loop β2 may participate in docking

The sequences of selected vertebrate glycoprotein hormones having lutropin, follitropin, and thyrotropin activities shown here illustrate the conserved nature of residues thought to participate in docking. Most lutropins and follitropins have a conserved valine at loop β2 residue 6 and most thyrotropins have an asparagine at this site. Other substitutions are known, however. Eel lutropins have a serine at this site. Alanine and isoleucine have also been reported, but these are rare and it is conceivable that they are sequencing artifacts for valine and asparagine, respectively. With the exception of a glutamine at positions 16 (lutropins and follitropins) and 18 (thyrotropins) most residues in loop β2 are not highly conserved among vertebrate glycoprotein hormones.

Species	Accession number	Sequence of loop β2
Lutropins and choriogonadotropins		
Human (CG)	AAL69704	ptmtr**V**lggvlpalpqvv
Human (LH)	NP_000885	ptmmr**V**lqavlpplpqvv
Marsupial	AAL13337	psmvr**V**lpaalppgpqlv
Chicken	a61091	rtrep**V**yrsplgpppqsa
Japanese toad	BAB93552	wakdp**V**yktalaavkqki
Striped bass	i50994	itkdp**V**ikipfsnvyqhv
Goldfish	q98849	ltkep**V**ykspfstvyqhv
Rainbow trout	BAB17687	vtkep**V**fkspfstvyqhv
Sturgeon	cAB93502	ptkdp**V**fksalstvqqhv
Spotted catshark	cac43236	ptkes**V**ykspllsvyqhv
Follitropins		
Human	np0005501	ytrdl**V**ykdparpkiqkt
Marsupial	aak92541	htrdl**V**ykepirpniqka
Chicken	aa199279	ftrdp**V**ykyppvssvqqi
Japanese toad	bab93558	dtkdp**N**lkyphksekqrv
Stripped bass	AAC38035	yhedl**V**yishyerpeqri
Goldfish	q98848	ktqes**V**yrsplmlsyqnt
Rainbow trout	BAB17686	ettdl**N**yqstwlprsqgv
Sturgeon	cab93504	ltqad**V**ykssislytqlv
Spotted catshark	cac43235	ftkdp**V**ckhsmasiyqdi
Thyrotropins		
Human	aab30828	mtrdi**N**gklflpkyalsqdv
Marsupial	AAL05938	mtrds**N**gklflpksalsqdv
Chicken	o57340	mtrds**N**gkklllksalsqnv
Japanese toad	bab93563	ktmdp**N**vkgrqlktlsnqnv
Goldfish	BAA20081	fsrds**N**vkelvgarflvqrg
Rainbow trout	p37240	ysrds**N**mkelagprfliqrg
Sturgeon	cab93505	vtrdv**N**lksllpksalsqss

heterodimer, this disulfide would limit motions of loop α2 that destabilize hydrogen bonds between loop α2 and the β-subunit. The stability of these hydrogen bonds explains why the heterodimer dissociates at low pH or in urea but not in the presence of most detergents. It will also account for the finding that changes in the size of the tensor loop disrupt heterodimer formation (15). Hydrogen bonds formed following threading of these subunits would stabilize βCys^{100} in a position in which it is less likely to reform the tensor disulfide.

The tensor disulfide appears to remain intact during assembly that occurs by the wraparound pathway (Fig. 3). Consequently, hydrogen bonds can be formed between residues in loop α2 and the region of the seatbelt at the junction of the tensor loop and its strap similar to those observed in the heterodimer. This would stabilize the position of the NH_2-terminal half of the seatbelt, thereby increasing the probability that hCG β-subunit residue βCys^{110} is near βCys^{26}, a phenomenon needed for seatbelt latch disulfide formation. Experimental support for this notion (Table V) is provided by the finding that the disruption of the tensor disulfide reduced the ability of antibody B111 to recognize heterodimers that cannot latch their seatbelts. It also reduced the ability of the seatbelt to be cross-linked to a cysteine in the α-subunit (Table V), a phenomenon that occurs by the wraparound pathway.

Rate-limiting Steps in Heterodimer Assembly—An outline of the major steps in hCG assembly (Fig. 4) summarizes what we have learned about hCG assembly in the ER. The subunits dock reversibly before and after the seatbelt is latched, but most assembly occurs by a threading mechanism while the seatbelt is latched and the tensor disulfide is disrupted. Which of these steps is rate-limiting? We suggest that disruption of the tensor disulfide is the principle rate-limiting step in hCG assembly. The finding that docking is readily reversible before and after the seatbelt is latched shows that it is not likely to be rate-limiting for assembly by either a threading or wrapping mechanism. Several findings suggest that disruption of the tensor disulfide is rate-limiting for the threading pathway. First, the tensor disulfide forms before the seatbelt is latched and facilitates latching (18). Second, disruption of the tensor disulfide is accompanied by the formation of several contacts between loop α2 and the β-subunit that would be expected to stabilize the docked complex and to facilitate threading (Fig. 2, *right*). And third, disruption of the tensor disulfide is known to occur during hCG, hFSH, and hTSH assembly in the ER (18) and to accelerate assembly dramatically *in vitro* (16). Disruption of the tensor disulfide could occur before or after the subunits dock, but we are unable to distinguish these possibilities. In contrast to the hCG, the rate-limiting steps in the assembly of teFSH remain unclear. Disruption of the tensor disulfide interferes with assembly by the wraparound pathway, possibly because it interferes with docking and latching (Table V, data rows 4–6). Conceivably, teFSH assembly is limited by the rate at which the seatbelt is wrapped around loop α2 and latched. Premature latching of the seatbelt would significantly deplete the concentration of β-subunit in the ER.

Implications for the Assembly of Other Vertebrate Glycoprotein Hormones—Observations described here and in the accompanying articles (17–19) will explain the ER assembly of hCG, hFSH, hTSH, and teFSH. Based on these findings, we anticipate that most glycoprotein hormone heterodimers in which the seatbelt is latched to loop β1 are assembled by threading mechanisms. Nonetheless, it is conceivable that the composition of the seatbelt can retard threading as was found when the hCG seatbelt was replaced by its salmon FSH counterpart (19). Thus, many exceptions may be found to the hypothesis that a threading pathway is used to assemble most vertebrate glycoprotein heterodimers in which the seatbelt is latched to a cysteine in loop β1. We are currently attempting to identify factors that can limit threading by systematically studying the influence of several vertebrate hormone seatbelts on heterodimer assembly. Preliminary findings suggest that the seatbelts of LH and FSH of the shark, the most ancient species for which sequences are available, permit efficient assembly by a threading mechanism.

Acknowledgments—We thank Dr. William Munroe (Hybritech, Incorporated, San Diego, CA, a subsidiary of Beckman Coulter, Inc.) for antibodies A113 and B111, Dr. Robert Canfield (Columbia University, New York) for antibody B101, and Dr. Robert Campbell (Serono Reproductive Biology Institute, Rockland, MA) for purified recombinant hCG.

REFERENCES

1. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature* **369,** 455–461
2. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) *Structure* **2,** 545–558
3. Fox, K. M., Dias, J. A., and Van Roey, P. (2001) *Mol. Endocrinol.* **15,** 378–389
4. Ruddon, R. W., Sherman, S. A., and Bedows, E. (1996) *Protein Sci.* **8,** 1443–1452
5. Xing, Y., Lin, W., Jiang, M., Myers, R. V., Cao, D., Bernard, M. P., and Moyle, W. R. (2001) *J. Biol. Chem.* **276,** 46953–46960
6. Suganuma, N., Matzuk, M. M., and Boime, I. (1989) *J. Biol. Chem.* **264,** 19302–19307
7. Heikoop, J. C., van den Boogaart, P., Mulders, J. W. M., and Grootenhuis, P. D. J. (1997) *Nat. Biotech.* **15,** 658–662
8. Einstein, M., Lin, W., Macdonald, G. J., and Moyle, W. R. (2001) *Exp. Biol. Med.* **226,** 581–590
9. Aloj, S. M., Edelhock, H., Ingham, K. C., Morgan, F. J., Canfield, R. E., and Ross, G. T. (1973) *Arch. Biochem. Biophys.* **159,** 497–504

10. Huang, J., Chen, F., and Puett, D. (1993) *J. Biol. Chem.* **268,** 9311–9315
11. Slaughter, S., Wang, Y. H., Myers, R. V., and Moyle, W. R. (1995) *Mol. Cell. Endocrinol.* **112,** 21–25
12. Chen, F., and Puett, D. (1991) *J. Biol. Chem.* **266,** 6904–6908
13. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50,** 465–495
14. De Beer, T., Van Zuylen C. W., Leeflang, B. R., Hard, K., Boelens, R., Kaptein, R., Kamerling, J. P., and Vliegenthart, J. F. (1996) *Eur. J. Biochem.* **241,** 229–242
15. Lin, W., Ransom, M. X., Myers, R. V., Bernard, M. P., and Moyle, W. R. (1999) *Mol. Cell. Endocrinol.* **152,** 91–98
16. Xing, Y., Williams, C., Campbell, R. K., Cook, S., Knoppers, M., Addona, T., Altarocca, V., and Moyle, W. R. (2001) *Protein Sci.* **10,** 226–235
17. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35426–35436
18. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35437–35448
19. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35449–35457

The Journal of Biological Chemistry

ASBMB

jbc ONLINE

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE IV

Formation of the α5-β5 intersubunit cross-link rescued the formation of heterodimers containing s/hCGβ-Nt,SB

COS-7 cells were incubated with α-Q5C and either s/hCGβ-Nt or s/hCGβ-Nt,SB to determine the influence of the hCG and salmon seatbelt on latching the seatbelt to loop β1. The stabilities of the heterodimers were measured following an incubation at pH 2, 37 °C, for 30 min or overnight. Values shown are means of triplicate transfections ± S.E. Values for heterodimers containing the native human α-subunit and s/hCGβ-Nt,SB were estimated following concentration of the media 10-fold and are expressed here in units that reflect the original concentration of the medium. The reduction in the amount of this heterodimer caused by pH 2, 37 °C treatment show that its seatbelt is latched to βCys^{5}. The seatbelt in the heterodimer containing α-Q5C and s/hCGβ-Nt was recognized by antibody B111 (not shown) indicating that it was latched to βCys^{26}. A similar quantity of acid-stable heterodimer was produced when s/hCGβ-Nt,SB was co-transfected with α-Q5C. Since B111 does not recognize heterodimers containing the salmon seatbelt, we were unable to determine the location of the seatbelt latch site in this heterodimer directly. We expect that it is latched to βCys^{26}, however, due to the fact that elimination of this cysteine enhances the assembly of heterodimers containing the native human α-subunit and s/hCGβ-Nt,SB,C26A (Table I). Thus, we anticipate that most heterodimers containing α-Q5C and s/hCGβ-Nt,SB are stabilized by an α5-β5 cross-link and that much less, if any, is stabilized by the formation of a cross-link between βCys^{110} at the end of the seatbelt and αCys^{5}.

Data row	Analog	Total heterodimer	pH 2 stable, 30 min	pH 2 stable, 16 h
		ng/50 μl ± S.E.	*% total ± S.E.*	
1	α-Q5C+s/hCGβ-Nt	5.48 ± 0.44	99.4 ± 3.7	103.7 ± 4.7
2	α-Q5C + s/hCGβ-Nt,SB	4.73 ± 0.09	112.9 ± 8.3	99.2 ± 4.6
3	α + s/hCGβ-Nt,SB	0.12 ± 0.01	67.5 ± 3.1	34.9 ± 3.7

TABLE V

Influence of the salmon seatbelt tensor loop and strap on heterodimer assembly

The indicated β-subunit analogs were co-transfected into COS-7 cells with the native α-subunit. Production of heterodimer was monitored in the medium three days later. The stability of the heterodimer was measured following an incubation at pH 2, 37 °C for 30 min. Values shown are mean ± S.E. for triplicate transfections.

Row	β-Subunit	Total	% Stable
		ng/50μl	*% total ± S.E.*
1	hCGβ	2.34 ± 0.28	0.4 ± 0.3
2	s/hCGβ-SB	0.39 ± 0.12	0.2 ± 0.1
3	s/hCGβ-SB,L92K	0.67 ± 0.12	0.7 ± 0.3
4	s/hCGβ-TL	2.27 ± 0.26	0.3 ± 0.2
5	s/hCGβ-St	0.28 ± 0.07	5.2 ± 0.5

TABLE VI

Stability of heterodimers containing the salmon seatbelt strap region following co-expression with α-S43C and α-S92C

The indicated β-subunit analogs were co-transfected into COS-7 cells with the α-subunit analog αS43C and αS92C, respectively. Production of heterodimer was monitored in the medium three days later in A113/^{125}I-B111 sandwich immunoassays. The stability of the heterodimer was measured following an incubation at pH 2, 37 °C for 30 mins. Values shown are mean ± S.E. for triplicate incubations.

Data row		ng/50 μl ± S.E.	% Stable ± S.E.
α-S43C			
1	+hCGβ	7.27 ± 0.49	10.3 ± 0.6
2	+s/hCGβ-St	1.58 ± 0.04	44.4 ± 1.4
3	+s/hCGβ-St,C93A	0.44 ± 0.03	102.9 ± 1.2
4	+s/hCGβ-St,C93A,C100A	Not detected	Not done
α-S92C			
5	+hCGβ	7.67 ± 0.44	3.8 ± 0.1
6	+s/hCG-St	2.09 ± 0.10	10.1 ± 0.9
7	+s/hCGβ-St,C93A	0.36 ± 0.03	113.4 ± 5.3
8	+s/hCGβ-St,C93A,C100A	Not detected	Not done

teine added to loop 2 of the α-subunit and one of the tensor cysteines. This type of cross-link had been observed during the assembly of hCG (20). As expected for assembly that occurs by a threading mechanism, a substantial fraction of the heterodimer formed when α-S43C was co-expressed with s/hCGβ-St was stable at pH 2, 37 °C (Table VI, data row 2). This suggested that a cross-link had formed between one of the tensor cysteines in s/hCGβ-St and αCys^{43}. The finding that a much larger fraction of the heterodimer containing s/hCGβ-St was cross-linked than that containing hCGβ supported the hypothesis that threading had been delayed. This notion was also supported by the finding that expression of s/hCGβ-St with α-S92C, a region of the α-subunit that is not threaded beneath the seatbelt, led to lesser amounts of cross-link (Table VI, data row 6). The hypothesis that these disulfides involved a tensor cysteine is consistent with the finding that co-expression of either α-S43C or α-S92C with s/hCGβ-St,C93A, an analog that has an unpaired tensor cysteine at βCys^{100} led to the formation of heterodimers that were completely acid stable (Table VI, data rows 3 and 7). In contrast, co-expression of α-S43C and α-S92C with s/hCGβ-St,C93A,C100A, an analog that lacks both tensor cysteines resulted in the formation of only trace amounts of heterodimers (Table VI, data rows 4 and 8).

DISCUSSION

Mechanisms of Glycoprotein Hormone Assembly in Vertebrates—Because of the tendency of the seatbelt to be latched to a cysteine in loop β1 before the subunits dock, we anticipate that most vertebrate glycoprotein hormones are assembled by a threading mechanism. Indeed, as shown here, even the salmon FSH seatbelt tends to be latched to a cysteine in loop β1 when it has a choice of seatbelt latch sites. When the seatbelt can only be latched to an NH_2-terminal β-subunit site, however, as it is in the case for salmon FSH and other related teleost species (Table VII), heterodimer assembly can occur by a wraparound mechanism, but not by threading. This appears due to the combination of the low ability of the salmon seatbelt to be latched to the NH_2 terminus and the difficulty of threading loop α2 beneath these seatbelts once they are latched. The finding that the salmon seatbelt can impede threading when it is latched to loop β1 suggests that some glycoprotein hormones may be assembled by a wraparound pathway even when their seatbelts are latched to loop β1. We would expect this process to be inefficient, however.

Implications of these Observations for the Evolution of teFSH and the Interaction of teFSH with Piscine FSH Receptors—The finding that hCG analogs which have the teFSH folding pattern are assembled by a wraparound mechanism suggest that hormones such as salmon FSH are also assembled in this fashion. Why would the wraparound pathway, an assembly mechanism that appears to be relatively inefficient (19, 20), be used to produce hormones that are usually thought to be critical for the reproduction of vertebrates? The wraparound pathway permits formation of heterodimers that cannot be assembled by threading. As such, it would have facilitated natural experimentation with the seatbelt during co-evolution of these heterodimeric ligands and their receptors. The seatbelt is responsible for much of the influence of the hormone-specific β-subunit on biological activity (5–8) and, as shown here, the efficiency of threading. When changes in the β-subunit create seatbelts that block threading, the use of the wraparound mechanism offers the organism a mechanism of producing het-

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

TABLE VII
Migration of the seatbelt latch site in fish

We anticipate that all glycoprotein hormones originated from a precursor in which the seatbelt was latched to a cysteine in β1. This would permit assembly by either a threading or wraparound mechanism. Selection pressure to optimize reproduction and thyroid function led to the duplication and reduplication of the β-subunit to create β-subunits found in lutropins, follitropins, and thyrotropins. Subsequent divergence of the resulting β-subunits occurred in response to selection pressures on reproduction and development. The seatbelt is the portion of the β-subunit that has the greatest influence on hormone activity (5–8), making it more sensitive to selection pressure than other parts of the protein. Its roles in heterodimer assembly and stability would also have influenced β-subunit evolution. Mutations of the seatbelt that prevented assembly would have been lost regardless of their abilities to regulate receptor function. The existence of the wraparound assembly mechanism would have permitted mutations to the seatbelt that prevented threading, even though wrapping appears to be much less efficient than threading. In part, this is due to the tendency of the seatbelt to become latched to the β-subunit before it docks with the α-subunit. Factors that drove the evolution of teFSH remain unknown. In teleosts, these are likely to have involved seatbelt mutations that affected the interaction of teleost FSH with FSH, LH, and TSH receptors. Relocation of the seatbelt to the NH_2 terminus of the β-subunit would be expected to disrupt high affinity interactions between the COOH terminal end of the seatbelt and any of these receptors. Based on the studies of Yan *et al.* (18), which show that salmon FSH does not bind the salmon LH receptor, we propose that the evolution of the teFSH structure was driven by the need to reduce cross-reactions with the LH receptor and/or to increase the stability of the heterodimer. Shown here (single letter code) are parts of the β-subunit corresponding to the NH_2 terminus (Nt) and seatbelt along with the residue corresponding to hCG βCys^{26}, the β1 seatbelt latch site. The signal sequence cleavage site has not been determined experimentally in all cases and the NH_2-terminal residues shown here were determined by analogy to known sequences. The cleavage sequence for s/hCGβ-Nt,SB,C26A was assumed to be identical to that of hCGβ, which would give it an additional serine residue that is not found in the salmon FSH β-subunit. It should be noted that the signal peptides of many vertebrate FSH β-subunits contain additional cysteines. Therefore, the introduction of an NH_2-terminal cysteine in the β-subunit may have arisen from a modification of the signal peptide cleavage site. The assembly pathway is known only for hCG, hFSH, hTSH, and salmon FSH. That for the remaining β-subunits is inferred from these. The term "unknown-T" is used to indicate that two pathways are possible but that threading appears to be most likely.

Species and β-subunit	Acquisition number	Assembly pathway	Amino terminus	Residue in β1	Seatbelt region	
					Tensor	Strap
Human hCGβ	NP_000728	Threading	SKEPLRPRC	C	AL CRRSTTDC	GGPKDHPLTC
Human FSHβ	1FL7B	Threading	NSC	C	GK CDSDSTDC	TVRGLGPSYC
Human TSHβ	AAB30828	Threading	FC	C	GK CNTDYSDC	IHEAIKTNYC
Shark FSHβ	CAC43235	Threading	NRC	C	GM CNTETTDC	TVSAMEPTHC
Japanese eel	Q9YGK3	Threading	RA**S**TSC	C	SK CNTDSTDC	GPLNTEVSGC
Conger eel	CAB93518	Wrapping	RA**C**SSC	**W**	SR CNTNSTDC	GQLNTEASGC
Black carp FSHβ	AAK07415	Threading	GSE**F**RSSC	C	SK CNSDIADC	GVLSQQTSSC
Common carp FSHβ	O13050	Unknown-T	GSE**C**RSSC	C	SK CNSDITDC	GALSQQTLSC
Goldfish FSHβ	Q98848	Unknown-T	GSE**C**RSSC	C	SK CNSDITDC	GVLSQQTLGC
Chum salmon FSHβ	P10257	Wrapping	GTE**C**RYGC	**S**	IK CKTDNTDC	DRISMATPSC

erodimers that may enable it to reproduce.[2]

The evolutionary pressures which drove the change in glycoprotein hormone topology that created teFSH are enigmatic, particularly because hormones having this structure appear to be more difficult to assemble. One factor that may have contributed to the development of teFSH is its increased stability, which may offset the additional difficulty of assembling the heterodimer. More likely, the changes in the teFSH β-subunit occurred during the continual co-evolution of the gonadotropins and their receptors (6). Both salmon LH and salmon FSH interact with the salmon FSH receptor (18), indicating that some of the actions of salmon FSH can be replaced by salmon LH. In contrast, salmon FSH does not interact with the salmon LH receptor, a property that may reflect its altered seatbelt latch site. The observation that the FSH strap region of the seatbelt occupies two very different positions in the Japanese eel and the conger eel (Table VII) suggests that the strap may not participate in high affinity contacts with the FSH receptor in either of these related species. If the strap region contributed to the ability of FSH to bind to LH receptors when it was latched to loop β1, changing its position to the NH_2 terminus would reduce binding to the LH receptor without affecting binding to the FSH receptor.

The most ancient species for which a sequence of the FSH β-subunit is known is the shark. The seatbelt of this follitropin appears to be latched to a site in loop β1. We have found that hCG-shark FSH chimeras containing the shark FSH seatbelt are assembled into heterodimers readily and that this is not affected by adding the salmon NH_2-terminal seatbelt latch site.[3] The finding that the shark FSH seatbelt is readily latched to loop β1 and that it does not inhibit threading suggests strongly that shark FSH is assembled by a threading mechanism similar to β-subunit analog s/hCGβ-Nt (Table II, row 3). The FSH β-subunit in goldfish and the common carp appears to have two seatbelt latch sites, one in the NH_2 terminus and one in loop β1, but it is not known which of these latch sites are used (Table VII). Indeed, whereas it is conceivable that these fish produce two forms of FSH, we expect that similar to s/hCGβ-Nt most of their FSH will be assembled by a threading mechanism and its seatbelt will be latched to loop β1. This is supported by the finding that black carp FSH, which has a seatbelt that is similar to that of the common carp and goldfish, appears to have only the seatbelt latch site in loop β1 (Table VII). Thus, it would be expected that this heterodimer forms by threading. The presence of two potential latch sites within the goldfish and common carp β-subunits suggests that the precursor of teFSHβ may have also had two potential latch sites. Based on our finding that s/hCGβ-Nt,SB is assembled into heterodimers poorly, we expect that the site in loop β1 was eliminated to enhance heterodimer assembly. Loss of the loop β1 latch site would have reduced premature latching of the seatbelt, thereby facilitating assembly by the wraparound pathway.

The Strategies Devised to Study the Assembly of hCG and Analogs Having the Folding Pattern of teFSH Can be Used to Study the Assembly of Other Vertebrate Glycoprotein Hormones—The amino acid sequences of several vertebrate hormones have been reported, but it remains to be determined how

[2] Fish gonadotropins often differ from their mammalian counterparts. In many cases, the piscine lutropin, which is also known as GTH-II is capable of interacting with LH and FSH receptors. This may have also had a role during the evolution of teFSH.

[3] M. P. Bernard, R. V. Myers, D. Cao, and W. R. Moyle, unpublished data.

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

ASBMB The Journal of Biological Chemistry jbc

these are assembled. Considerable variations occur in the sizes of the α- and β-subunit cores and the seatbelt regions of many glycoprotein hormones, particularly in fish. These have the potential to provide new insights into the mechanisms of protein folding within the endoplasmic reticulum. We anticipate that the approaches described here for studying the assembly of teFSH will be applicable to studies of the assembly of glycoprotein hormones from most vertebrates including fish. The most important aspect of our approach is the use of α- and β-subunit analogs that contain unpaired cysteines. Although we took advantage of a well characterized panel of monoclonal antibodies to hCG, we anticipate that any procedure capable of measuring the amounts of heterodimers produced following transfection of cells with subunit analogs containing appropriate unpaired cysteines would suffice. This includes the use of epitope tagged α- and β-subunit analogs. Many of the studies described in this and in the accompanying articles (19, 20) were performed to check the internal consistency of our findings. We anticipate that only a few of the analogs that we produced and characterized would be required to distinguish most assembly pathways. These would include analogs corresponding to α-Q5C, α-S43C, α-S92C, and hCGβ-C26A.

Acknowledgments—We thank Dr. William Munroe (Hybritech, Incorporated, San Diego, CA, a subsidiary of Beckman Coulter, Inc.) for antibodies A113 and B111, and Dr. Robert Campbell (Serono Reproductive Biology Institute, Rockland, MA) for purified recombinant hCG. We thank Drs. Robert Campbell and Penny Swanson (Northwest Fisheries Science Center, National Oceanic and Atmospheric Administration, Seattle, WA) for valuable suggestions.

REFERENCES

1. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50,** 465–495
2. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature* **369,** 455–461
3. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) *Structure* **2,** 545–558
4. Fox, K. M., Dias, J. A., and Van Roey, P. (2001) *Mol. Endocrinol.* **15,** 378–389
5. Campbell, R. K., Dean Emig, D. M., and Moyle, W. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 760–764
6. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) *Nature* **368,** 251–255
7. Dias, J. A., Zhang, Y., and Liu, X. (1994) *J. Biol. Chem.* **269,** 25289–25294
8. Grossmann, M., Szkudlinski, M. W., Wong, R., Dias, J. A., Ji, T. H., and Weintraub, B. D. (1997) *J. Biol. Chem.* **272,** 15532–15540
9. Li, M. D., and Ford, J. J. (1998) *J. Endocrinol.* **156,** 529–542
10. Garcia-Hernandez, M. P., Koide, Y., Diaz, M. V., and Kawauchi, H. (1997) *Gen. Comp. Endocrinol.* **106,** 389–399
11. Swanson, P., Suzuki, K., Kawauchi, H., and Dickhoff, W. W. (1991) *Biol. Reprod.* **44,** 29–38
12. Aloj, S. M., Edelhock, H., Ingham, K. C., Morgan, F. J., Canfield, R. E., and Ross, G. T. (1973) *Arch. Biochem. Biophys.* **159,** 497–504
13. Xing, Y., Lin, W., Jiang, M., Myers, R. V., Cao, D., Bernard, M. P., and Moyle, W. R. (2001) *J. Biol. Chem.* **276,** 46953–46960
14. Heikoop, J. C., van den Boogaart, P., Mulders, J. W. M., and Grootenhuis, P. D. J. (1997) *Nat. Biotech.* **15,** 658–662
15. Chen, F., and Puett, D. (1991) *J. Biol. Chem.* **266,** 6904–6908
16. Slaughter, S., Wang, Y. H., Myers, R. V., and Moyle, W. R. (1995) *Mol. Cell. Endocrinol.* **112,** 21–25
17. Campbell, R. K., Bergert, E. R., Wang, Y., Morris, J. C., and Moyle, W. R. (1997) *Nature Biotech.* **15,** 439–443
18. Yan, L., Swanson, P., and Dickhoff, W. W. (1992) *Biol. Reprod.* **47,** 418–427
19. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35426–35436
20. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35437–35448

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

The Journal of Biological Chemistry

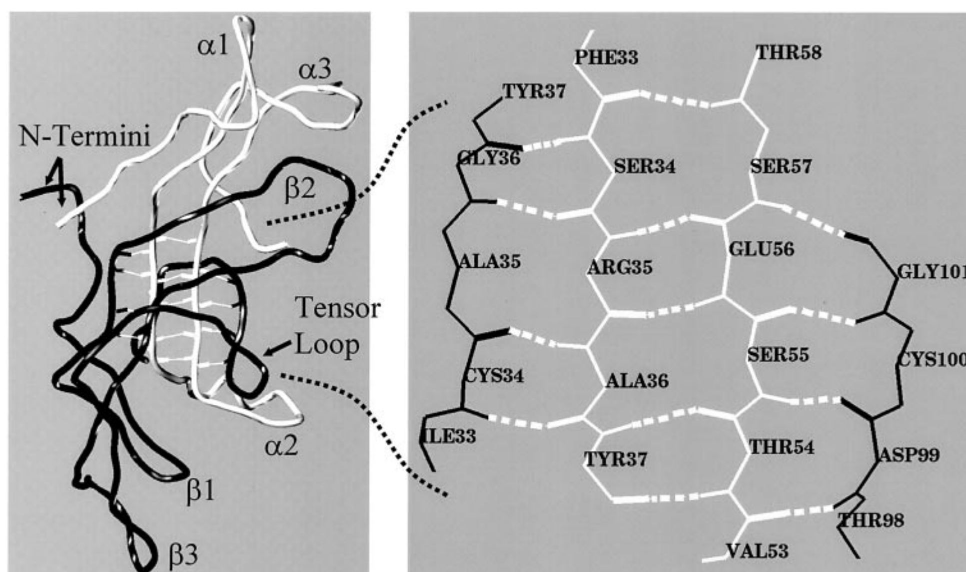

FIG. 6. **Hydrogen bonds in hCG that are expected to drive threading and to stabilize the β93-β100 tensor disulfide.** The orientation of the heterodimer (*left panel*) is positioned to illustrate hydrogen bonds that stabilize a β-sheet structure formed by residues in both subunits (1). Hydrogen bonds that stabilize other portions of the heterodimer have been omitted for clarity. Color code: *white*, α-subunit; *dark gray*, β-subunit; *thin white lines* (*left panel*) or *thick broken lines* (*right panel*), hydrogen bonds. A similar arrangement is seen in hFSH (3). The corresponding hFSH β-subunit residues are: βTrp^{27},βCys^{28},βAla^{29},βGly^{30},βTyr^{31} and βThr^{92},βAsp^{93},βCys^{94},βThr^{95}.

The transient nature of the threading process precluded its direct measurement. The notion that the tensor disulfide is disrupted during threading of hCG, hFSH, and hTSH is supported by the observation that one of the tensor cysteines becomes disulfide bridged to the α-subunit during the assembly of heterodimers that contain an unpaired α-subunit cysteine. Several observations support the notion that the tensor disulfide is involved in this cross-link. First, either tensor cysteine can become cross-linked to cysteines that have been substituted for several different α-subunit residues (Table II). This shows that the cross-link is feasible. Second, at least one tensor cysteine was required to form the cross-link. Replacing both tensor cysteines with alanine prevented the cross-link from forming. Third, only those cysteines in the α-subunit that are near a tensor cysteine were found to participate in the intersubunit cross-link. None of the analogs that contained a cysteine at a more remote site became cross-linked. Fourth, formation of this cross-link was detected only when the α-subunit was co-transfected with β-subunits that were capable of latching their seatbelts. And finally, all the cross-linked analogs tested were detected by B111, a phenomenon that would not have been detected if seatbelt residue βCys^{110} were latched to any other cysteine in the molecule. This showed that the end of the seatbelt was latched to βCys^{26}, not to the cysteine in the α-subunit.

We considered the possibility that the intersubunit cross-link was formed after the heterodimer had been assembled rather than during the process of threading. The finding that the intersubunit disulfide could be readily disrupted by mild reduction excluded this possibility (Table VI). These observations revealed that the cross-link is less stable than the tensor disulfide and that the cross-linked analogs are likely to be unstable intermediates that became trapped kinetically during threading. As a result, there would be no tendency to form a stable intersubunit disulfide after the tensor disulfide had been formed even in the reducing environment in the endoplasmic reticulum. The notion that cross-linked heterodimers are unstable intermediates is also consistent with the finding that only a fraction of the total heterodimers contained a cross-link.

Differences in the Redox Potential of the Tensor Disulfide in the Free β-Subunit and the Heterodimer Appear to Drive Glycoprotein Hormone Assembly by the Threading Pathway—The tensor disulfide is less stable in the free subunit than in the heterodimer (Fig. 5) and its disruption would facilitate threading of α2 by increasing the space that is available for passage of the glycosylated end of α2. Indeed, the latter may have the greatest requirement for space because the rate of assembly in the absence of this oligosaccharide exceeds that in its presence, a phenomenon that can be used to prepare hormone analogs lacking this oligosaccharide (16). Completion of assembly, a phenomenon that stabilizes the tensor disulfide, would impede passage of α2 beneath the seatbelt and contribute to heterodimer stability. This would explain the discrepancies noted in the kinetics of subunit association and heterodimer dissociation (17), the acceleration of hCG assembly by reducing agents (7), and the influence of protein-disulfide isomerase on assembly (8). Changes in the size and composition of the tensor loop, which would be expected to affect its formation, have been found to adversely affect heterodimer assembly by mammalian cells (18).

Why is the stability of the tensor disulfide in the heterodimer greater than that in the free β-subunit? The structure of the seatbelt in the free β-subunit has not been determined. Because this region of the seatbelt is not recognized by heterodimer-specific antibodies to epitopes that include portions of the NH_2-terminal end of the seatbelt (19), it is likely to have a different structure in the free β-subunit than in the heterodimer. In hCG, the backbone atoms of α-subunit residues αVal^{53},Ser^{55},Ser^{57} form hydrogen bonds with hCGβ-Asp^{99},Gly^{101} and possibly hCGβ-Thr^{97} (1, 2), a phenomenon that would constrain tensor cysteine hCGβ-Cys^{100} to a region nearby hCGβ-Cys^{93} (Fig. 6). In hFSH, these α-subunit residues form hydrogen bonds with hFSHβ-Ser^{91},Asp^{93},Thr^{95} (3), thereby constraining tensor cysteine hFSHβ-Cys^{94} nearby hFSHβ-Cys^{87}. Consequently, the reducing environment of the endoplasmic reticulum is less likely to disrupt a disulfide between the tensor cysteines in the heterodimer than in the free β-subunit. The α-subunit residues that participate in this network are held in a β-sheet with residues in α2 that are in contact with the β-subunit cystine knot (Fig. 6). Thus, assembly of the heterodimer stabilizes the position of the tensor loop relative to the β-subunit cystine knot, something that is un-

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

likely to occur in the free β-subunit.

Antibody Tools Are Useful for Structural Analyses—The studies described here and in the companion manuscripts (22–24) depended on the use of monoclonal antibodies to conformation-sensitive epitopes to evaluate the structures of various folding intermediates (20, 21). The most important of these was B111, the antibody that can detect an epitope that is formed when the hCG seatbelt is latched normally. This permitted studies in which various cysteines were allowed to compete with βCys^{26} and to determine when this disulfide was latched. Whereas it would have been preferable to use high resolution techniques such as crystallography or nuclear magnetic resonance spectroscopy to identify these intermediates, these techniques do not have the sensitivity required for the analysis of nanogram quantities of materials that can be produced readily. Even discounting the challenges of determining the structures of these intermediates by NMR and crystallography, it would have been cost prohibitive to make the larger amounts of materials required.

hCG contains a total of 11 disulfide bonds, making it possible that the introduction of cysteines into either subunit might disrupt one or more of these disulfides and alter its structure. With the exception of the seatbelt latch site and the apparent formation of disulfides between a tensor cysteine and the α-subunit observed during threading, we did not detect any signs that the cysteines we introduced or removed altered the structures of either subunit despite the fact that we analyzed a large number of cysteine containing constructs. Indeed, we sought to test the robustness of our approach by creating and testing a large panel of cysteine containing analogs. All of the observations that we made are internally consistent, a phenomenon that would be unlikely if some cysteine mutations had disrupted the structure of the hormone.

Our dependence on antibodies for these studies raises the possibility that we missed important assembly intermediates that are not recognized by any antibodies in our panel. For example, because all the antibodies used in these studies are conformation dependent, we would not observe heterodimer assembly that occurs before formation of the subunit cores, a phenomenon that depends on formation of their cystine knots. Whereas we cannot exclude the possibility that some assembly occurs by this route, the fact that we can account for most, if not all the heterodimer that is formed, makes it unlikely. As noted earlier (22), the difficulty of distinguishing dead end folding intermediates is one reason that we chose not to use pulse-chase methods for these studies. Finally, we attempted to exclude the possibility that our observations would be affected by changes to the protein that occur during secretion using analogs that are preferentially retained within the cell. We observed similar phenomena using analogs that lacked or contained the KDEL retention signal, indicating that our conclusions cannot be because of changes to the hormone that occur during its migration through the secretory pathway.

Acknowledgments—We thank Dr. William Munroe (Hybritech, Incorporated, San Diego, CA, a subsidiary of Beckman Coulter, Inc.) for antibodies A113, B111, B112, B603, and B806, Dr. Robert Canfield (Columbia University, New York) for antibody B101, and Dr. Robert Campbell (Serono Reproductive Biology Institute, Rockland, MA) for purified recombinant hCG and antibody B122.

REFERENCES

1. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) *Nature* **369,** 455–461
2. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) *Structure* **2,** 545–558
3. Fox, K. M., Dias, J. A., and Van Roey, P. (2001) *Mol. Endocrinol.* **15,** 378–389
4. Ruddon, R. W., Sherman, S. A., and Bedows, E. (1996) *Protein Sci.* **8,** 1443–1452
5. Xing, Y., Lin, W., Jiang, M., Myers, R. V., Cao, D., Bernard, M. P., and Moyle, W. R. (2001) *J. Biol. Chem.* **276,** 46953–46960
6. Pierce, J. G., and Parsons, T. F. (1981) *Annu. Rev. Biochem.* **50,** 465–495
7. Xing, Y., Williams, C., Campbell, R. K., Cook, S., Knoppers, M., Addona, T., Altarocca, V., and Moyle, W. R. (2001) *Protein Sci.* **10,** 226–235
8. Huth, J. R., Perini, F., Lockridge, O., Bedows, E., and Ruddon, R. W. (1993) *J. Biol. Chem.* **268,** 16472–16482
9. Campbell, R. K., Dean Emig, D. M., and Moyle, W. R. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 760–764
10. Munro, S., and Pelham, H. R. (1987) *Cell* **48,** 899–907
11. Suganuma, N., Matzuk, M. M., and Boime, I. (1989) *J. Biol. Chem.* **264,** 19302–19307
12. Moyle, W. R., Campbell, R. K., Myers, R. V., Bernard, M. P., Han, Y., and Wang, X. (1994) *Nature* **368,** 251–255
13. Grossmann, M., Szkudlinski, M. W., Wong, R., Dias, J. A., Ji, T. H., and Weintraub, B. D. (1997) *J. Biol. Chem.* **272,** 15532–15540
14. Dias, J. A., Zhang, Y., and Liu, X. (1994) *J. Biol. Chem.* **269,** 25289–25294
15. De Beer, T., Van Zuylen, C. W., Leeflang, B. R., Hard, K., Boelens, R., Kaptein, R., Kamerling, J. P., and Vliegenthart, J. F. (1996) *Eur. J. Biochem.* **241,** 229–242
16. Xing, Y., and Moyle, W. R. (2003) *Biochem. Biophys. Res. Commun.* **303,** 201–205
17. Strickland, T. W., and Puett, D. (1982) *J. Biol. Chem.* **257,** 2954–2960
18. Lin, W., Ransom, M. X., Myers, R. V., Bernard, M. P., and Moyle, W. R. (1999) *Mol. Cell. Endocrinol.* **152,** 91–98
19. Moyle, W. R., Matzuk, M. M., Campbell, R. K., Cogliani, E., Dean Emig, D. M., Krichevsky, A., Barnett, R. W., and Boime, I. (1990) *J. Biol. Chem.* **265,** 8511–8518
20. Cosowsky, L., Rao, S. N., Macdonald, G. J., Papkoff, H., Campbell, R. K., and Moyle, W. R. (1995) *J. Biol. Chem.* **270,** 20011–20019
21. Moyle, W. R., Campbell, R. K., Rao, S. N., Ayad, N. G., Bernard, M. P., Han, Y., and Wang, Y. (1995) *J. Biol. Chem.* **270,** 20020–20031
22. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35426–35436
23. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35449–35457
24. Xing, Y., Myers, R. V., Cao, D., Lin, W., Jiang, M., Bernard, M. P., and Moyle, W. R. (2004) *J. Biol. Chem.* **279,** 35458–35468

Downloaded from www.jbc.org at Princeton University Library on October 30, 2006

The Journal of Biological Chemistry