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Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

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

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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-

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[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.

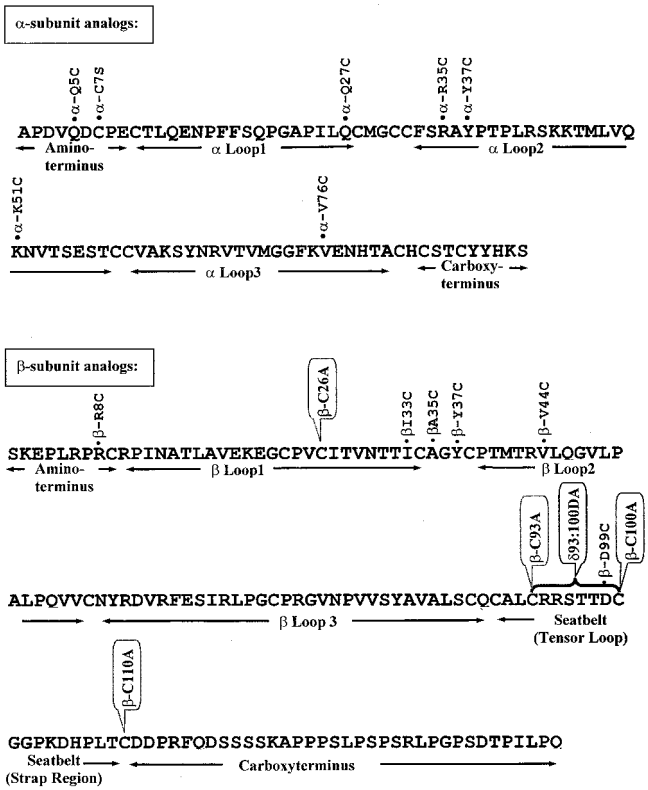

FIG. 1. **Constructs used in these studies.** The amino acid sequences of the constructs used in these studies are shown here. Residues *above* the sequence indicate amino acid substitutions. For example, α-Q5C represents an analog in which α-subunit residue αGln^5 is converted to cysteine. Several analogs 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-

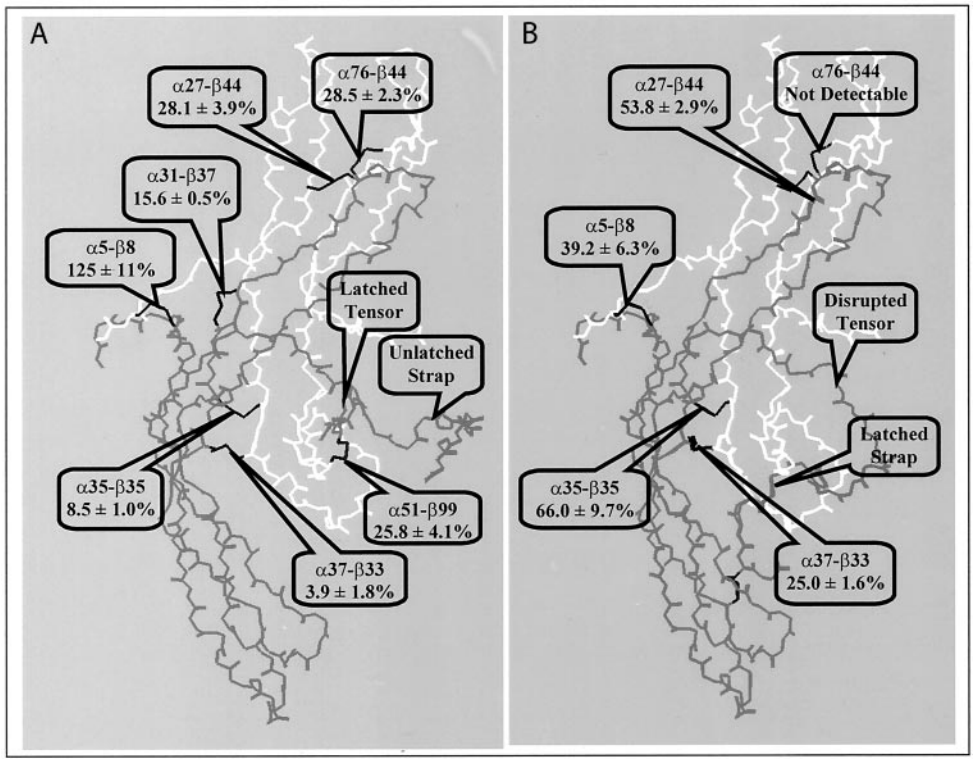

FIG. 2. **Contacts in the wraparound (*left panel*) and threading pathways (*right panel*).** Analogs of hCG β-subunit that lack the ability to form the seatbelt latch disulfide (β26-β110) do not combine with the α-subunit to form a stable heterodimer unless they contain an intersubunit disulfide cross-link. 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.

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 μl ± 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-

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

TABLE III
Influence of the seatbelt and Cootterminus 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

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).

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

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

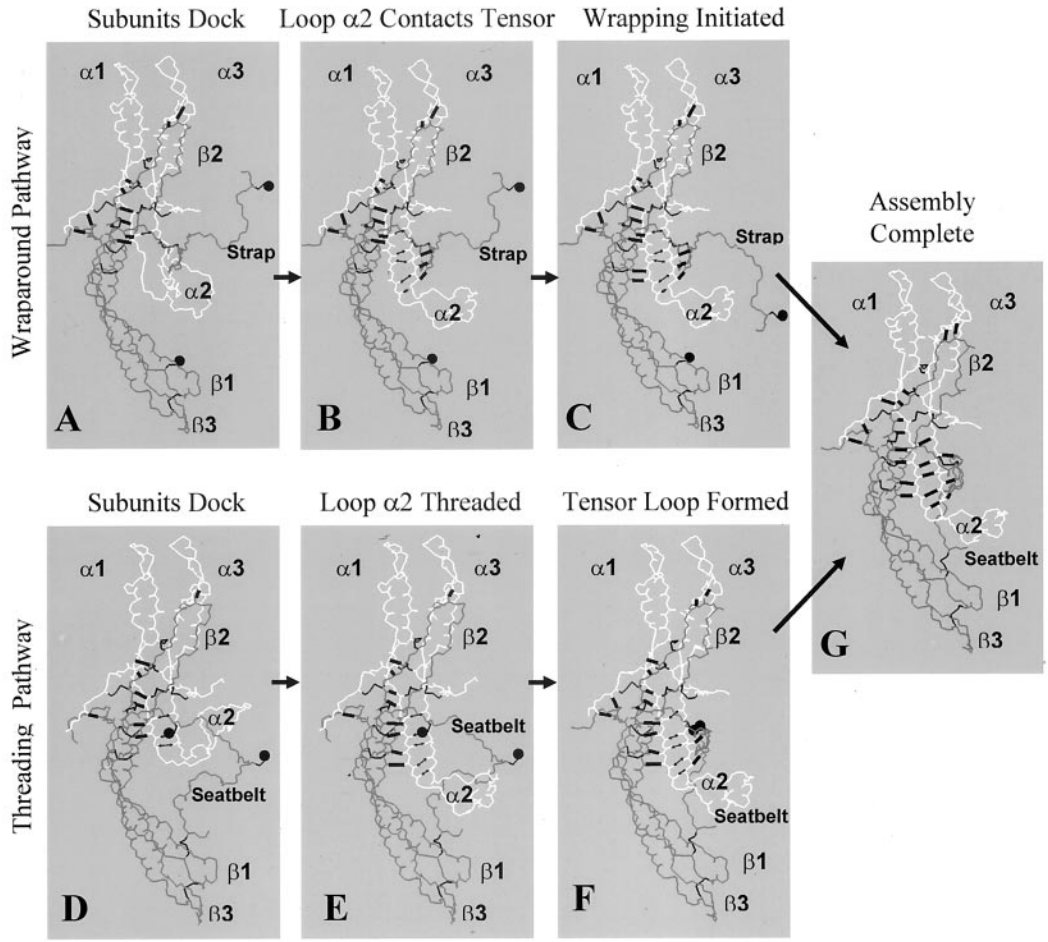

FIG. 3. **Models of assembly in the wraparound pathway (*upper panels*) and threading pathway (*lower panels*).** During subunit docking in both pathways contacts are formed between residues in the NH_2-terminal ends of both subunits and between NH_2-terminal residues in β-subunit loop β2 and α-subunit 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

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.

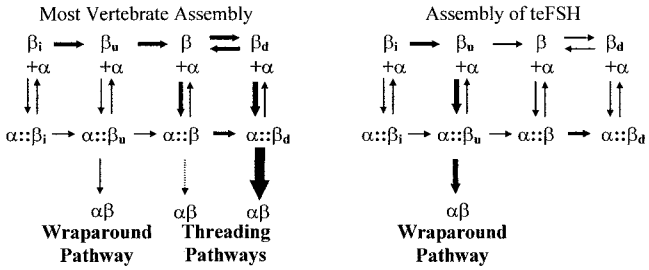

FIG. 4. **Summary of glycoprotein hormone assembly in the ER.** *Left diagram*, assembly of glycoprotein hormones in which the seatbelt is latched to loop β1. As reported here, the β-subunit can dock with the α-subunit before it has folded completely. Docking is reversible, however, and only a few forms 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 β1, 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 α2, 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; αβ, 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 β1. Thus, the reversibility of docking would promote latching the seatbelt to loop β1, 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 α2 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 α2 (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 α2, 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 α2 and parts of the β-subunit cystine knot, intrasubunit hydrogen bonds between several residues within the anti-parallel strands of loop α2 and its tip, and intersubunit hydrogen bonds between parts of loop α2 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 α2 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 $hCG\beta\text{-}Cys^{100}$ would be stabilized at a site near $hCG\beta\text{-}Cys^{93}$. 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

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.

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Glycoprotein Hormone Assembly in the Endoplasmic Reticulum: IV. PROBABLE MECHANISM OF SUBUNIT DOCKING AND COMPLETION OF ASSEMBLY

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