Glycoprotein Hormone Assembly in the Endoplasmic Reticulum

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

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 chorionicadotropin 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

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1 The abbreviations used are: α2, α-subunit loop 2; 61, β-subunit loop 1; BME, β-mercaptoethanol; IA, iodoacetate; hCG, human chorionicadotropin; hFSH, human follicle stimulating; hTSH, human thyrotropin.

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sequences of hCG

The amino acid sequences of the hCG in single letter format. The substitutions made are indicated thus, amino acid sequence and each mutant can be identified by its name.

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Thus, containing this signal (10). Thus, "the end of the seatbelt to the Leu) were fused to its COOH terminus. was converted to alanine and four additional residues (Lys-Asp-Glu-

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partic acid and alanine. Modeling suggested that this analog would when the tensor disulfide is present. The hFSH and hTSH hFSH-KDEL construct encodes hFSH-subunit residues 1–145 and KDEL fused to its carboxyl terminus. was secreted slowly (22) and are presumed to be retained in the ER.

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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

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.

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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 βCys26. Initial studies were designed to learn if the seatbelt would become latched in hCGβ-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 125I-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 βCys26 using a sandwich assay employing monoclonal antibodies B101 for capture and 125I-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 βCys26, 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 βCys93 and βCys100 (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.
Redox Control of Human Glycoprotein Hormone Assembly

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 2-subunit coding sequence either by changing the codons for either βCys⁹³ or βCys¹⁰⁰ to that for alanine (top panel) or by changing a codon for a residue in another part of the 2-subunit to that for cysteine (bottom panel). Constructs were expressed individually in COS-7 cells and 2-subunit analogs that were secreted or retained in the cells (KDEL constructs only) were measured in a sandwich immunoassay using B101 for capture and ¹²⁵I-B110 for detection relative to a purified hCG 2-subunit standard. B110 binding provided an estimate of the total amount of 2-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 ¹²⁵I-B111 for detection relative to the same hCG 2-subunit standard. The ability of the 2-subunit analogs to bind B111 relative to B110 provided an estimate of the fraction of the material in which the seatbelt is latched to βCys⁹³, 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 βCys⁹³ 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 βCys⁹³ as reflected by differences in their abilities to be recognized by B110 and B111 (Table I, rows 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 βCys⁹³ for formation of the seatbelt latch disulfide, not to a change in folding of the remainder of the 2-subunit. This is because changes in other parts of the 2-subunit would have disrupted the binding of antibodies B101 and B110 to the 2-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 βCys⁹³, βCys¹⁰⁰, and βCys¹¹⁰ are located near one another in the seatbelt. Therefore, before the seatbelt is latched, βCys¹¹⁰ is likely to be nearer tensor cysteines βCys⁹³ and βCys¹⁰⁰ than it is to βCys⁸⁶, the cysteine in loop β1 with which it will ultimately form the seatbelt latch disulfide. Consequently, βCys¹¹⁰ may compete with βCys⁹³ and βCys¹⁰⁰ for formation of the tensor disulfide. In analogs lacking both tensor cysteines, the seatbelt has no latch site other than βCys⁸⁶, 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, βCys¹¹⁰ has two potential latch sites, i.e. βCys⁸⁶ and either βCys⁹³ or βCys¹⁰⁰. The finding that the seatbelt was latched to βCys⁸⁶ in only a fraction of the β-subunit molecules that contained a single tensor cysteine suggests that βCys¹¹⁰ has little intrinsic tendency to be latched to βCys⁸⁶. Its proximity to βCys⁹³ and βCys¹⁰⁰ 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 βCys¹¹⁰, βCys⁹³, and βCys¹⁰⁰, βCys¹¹⁰ disulfides. Physical constraints on the positions of the latter disulfides cause them to remain near βCys¹⁰⁰ or βCys⁹³, which would enable them to be disrupted by a disulfide exchange involving βCys⁸⁶ or βCys⁹³, respectively. This would form the tensor disulfide and enable βCys¹¹⁰ to form the seatbelt latch disulfide with βCys⁸⁶. 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 βCys⁸⁶ (Table I, data rows 3–6). The probability that βCys¹¹⁰ forms a disulfide with βCys⁸⁶ before it forms a disulfide with βCys⁹³ or βCys¹⁰⁰ can be estimated from the fraction of hCGβ-C100A and hCGβ-C93A that is recognized by B111, respectively (Table I). Whereas βCys¹¹⁰ would also be capable of disrupting a disulfide between βCys⁹³ and βCys¹⁰⁰, 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 βCys⁸⁶ or βCys⁹³, 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 βCys¹¹⁰ at the end of the seatbelt is not constrained to a region near βCys⁸⁶. We tested the possibility that βCys¹¹⁰ scans the β-subunit to find its normal latch site by monitoring the abilities of cysteines added to the β-subunit to compete with βCys⁸⁶ for formation of the seatbelt latch disulfide. The rationale for this is described in

Alternatively, one could argue that seatbelt residue βCys¹¹⁰ forms a disulfide with the other cysteines in the β-subunit such as βCys⁹³, βCys⁷², 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 βCys⁷² or βCys⁹³ is absent (W. R. Moyle, unpublished data). Furthermore, βCys⁸⁶ and βCys⁹³ are further than βCys⁸⁶ from seatbelt residue βCys¹¹⁰, making them less likely to serve as seatbelt latch sites.
TABLE I

| Data row | hCGβ analog | Distance to βCys30 | Binding to B111 Relative to B110 |
|----------|-------------|---------------------|----------------------------------|
|          |             | Cys93 and Cys100    | C93A and C100A                    |
| 1        | hCGβ        | 0.16 ± 0.02         | 1.83 ± 0.16                      |
| 2        | hCGβ-KDEL   | 0.66 ± 0.01         | 1.29 ± 0.13                      |
| 3        | hCGβ-C93A   | 0.25 ± 0.01         |                                  |
| 4        | hCGβ-C93A-KDEL | 0.42 ± 0.07    |                                  |
| 5        | hCGβ-C100A  | 0.14 ± 0.02         |                                  |
| 6        | hCGβ-C100A-KDEL | 0.24 ± 0.05      |                                  |
| 7        | hCGβ-R8C    | 1.15 ± 0.09         | 0.78 ± 0.15                      |
| 8        | hCGβ-L16C   | 0.16 ± 0.02         | Not Done                         |
| 9        | hCGβ-E21C-KDEL | 0.63 ± 0.10     |                                  |
| 10       | hCGβ-E33C   | 0.36 ± 0.01         |                                  |
| 11       | hCGβ-A35C   | 0.44 ± 0.03         | 0.14 ± 0.01                      |
| 12       | hCGβ-A35C-KDEL | 0.29 ± 0.05     |                                  |
| 13       | hCGβ-F64C   | 0.31 ± 0.06         | Not Done                         |
| 14       | hCGβ-F64C-KDEL | 0.46 ± 0.01     |                                  |
| 15       | hCGβ-N77C   | 0.82 ± 0.14         | Not Done                         |
| 16       | hCGβ-N77C-KDEL | 0.47 ± 0.01     | 0.25 ± 0.03                      |
| 17       | hCGβ-A83C   | 0.37 ± 0.09         | Not Done                         |
| 18       | hCGβ-A83C-KDEL | 0.28 ± 0.01     | 0.53 ± 0.03                      |
| 19       | hCGβ-A91C   | 0.41 ± 0.08         | Not Done                         |
| 20       | hCGβ-S96C   | 0.21 ± 0.02         | Not Done                         |

There are no free cysteines in the hCG β-subunit and the distance illustrated is that between βCys93 and βCys100.

The total β-subunit was measured in sandwich assays employing antibodies B101 for capture (loop 2) and 125I-B110 (loops 1 and 3) or 125I-B112 (loop 3) for detection. Latching the seatbelt to βCys30 was determined using B101 for capture and 125I-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 βCys26 to the free cysteine. The endoplasmic reticulum-trapped analogs contained the KDEL sequence at their carboxyl termini. Values in the column denoted “Cys93 and Cys100” were for analogs that contain both tensor cysteines and that are expected to contain the tensor 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.

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 formation of the tensor disulfide.

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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 loop was intact, the seatbelt was latched properly, and the disulfide at the tips of loops β1 and β2 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 could 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 βCys100 (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 βCys100 (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 βCys100. 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 αTyr88 and αSer92.

Cysteines that had been added to the α-subunit were also capable of being cross-linked to βCys100, but this process appeared to be less efficient than formation of a cross-link with βCys100. 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 βCys100 than they are to βCys93. It may also suggest that α2 passes nearer βCys100 during threading. A notable exception to this generalization was the cysteine in α-N52C, αAsn62 appears to be nearer βCys93, 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 βCys110 and the cysteine added to the α-subunit was observed only when βCys93, the normal seatbelt latch site, had been replaced (5). Furthermore, we found that the seatbelts of analogs containing hCGβ-C93A were latched to βCys26 because these acid-stable heterodimers were recognized by B111 (Table II). Presumably these analogs contain a cross-link between βCys100 and the α-subunit. Some of the heterodimers containing hCGβ-C100A that we presumed to have a cross-link between βCys100 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
cross-linked heterodimer, we co-expressed several α-subunit an-
logs with hCG-β-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 β-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 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 con-
structs 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 substitu-
tion 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 dipetide, 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 het-
erodimer 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-β93-100D, hCGβ-β93-100DA, or
the KDEL derivatives of these analogs failed to produce heter-
dimers (Table V, data for KDEL derivatives not shown). Both
analogues 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
fraction of the heterodimers that contain an additional \( \alpha \)-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 heterodimers containing \( \beta \)-subunit analogs missing one tensor cysteine and \( \alpha \)-subunit analogs having an additional cysteine. Residues of the \( \alpha \)-subunit that appear to form an intersubunit disulfide cross-link when expressed with the native hCG \( \beta \)-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 \( \alpha \)-S92C, which does not form an intersubunit disulfide cross-link with hCG\( \beta \) but can form an intersubunit cross-link when expressed with an hCG \( \beta \)-subunit making one tensor cysteine is shown in dark grey with white lettering. Some \( \alpha \)-subunit analogs, such as \( \alpha \)-Q5C and \( \alpha \)-M71C, do not form an intersubunit disulfide with the hCG \( \beta \)-subunit during heterodimer assembly in the ER. These are shown in dark grey 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.

Fig. 4. Models depicting the positions of residues that appear to form an intersubunit disulfide with an hCG \( \beta \)-subunit tensor cysteine relative to those that do not. Co-expression of hCG \( \beta \)-subunit with some \( \alpha \)-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 \( \beta \text{Cys}^{38} \) in loop \( \beta 1 \), similar to that in hCG. The acid stabilities and B111 recognition properties of these heterodimers are similar to those of heterodimers containing \( \beta \)-subunit analogs missing one tensor cysteine and \( \alpha \)-subunit analogs having an additional cysteine. Residues of the \( \alpha \)-subunit that appear to form an intersubunit disulfide cross-link when expressed with the native hCG \( \beta \)-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 \( \alpha \)-S92C, which does not form an intersubunit disulfide cross-link with hCG\( \beta \) but can form an intersubunit cross-link when expressed with an hCG \( \beta \)-subunit making one tensor cysteine is shown in dark grey with white lettering. Some \( \alpha \)-subunit analogs, such as \( \alpha \)-Q5C and \( \alpha \)-M71C, do not form an intersubunit disulfide with the hCG \( \beta \)-subunit during heterodimer assembly in the ER. These are shown in dark grey 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.

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analogs in which the seatbelt is latched to 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 hCG analogs containing an additional cysteine was also stable at acid pH (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\(^{10}\) 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\(\mu\)l ± S.E. | % total ± S.E. | % stable ± S.E. |
| 1        | α                 | 5.46 ± 0.28 | 0.8 ± 0.3 | Not tested |
| 2        | α-Q5C             | 2.68 ± 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        | α-S-43C           | 6.98 ± 0.16 | 36.7 ± 1.8 | 41.6 ± 2.9 |
| 7        | α-K-44C           | 3.05 ± 0.01 | 11.0 ± 0.5 | 74.7 ± 0.7 |
| 8        | α-K-45C           | 1.35 ± 0.04 | 9.5 ± 0.6 | 55.4 ± 5.1 |
| 9        | α-T-46C           | 5.62 ± 0.27 | 32.7 ± 0.5 | 57.7 ± 0.2 |
| 10       | α-M47C            | 5.04 ± 0.56 | 22.2 ± 5.1 | 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-S-92C           | 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**

Constructs encoding the indicated α- and β-subunit analogs were transfected into COS-7 cells and heterodimer secreted into the medium was monitored by sandwich immunoassays employing an α-subunit antibody A113 for detection and radioiodinated β-subunit monovalent antibodies B110 or B111 for detection as described (22). The latter detects formation of a seatbelt latch between βCys\(^{10}\) and βCys\(^{19}\). Constructs used in transfection

| α-Subunit        | β-Subunit | Total dimer (B110) | pH 2 stable dimer (B110) | Total dimer (B111) | pH 2 stable dimer (B111) |
|------------------|-----------|--------------------|--------------------------|--------------------|--------------------------|
| 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           | hCGγ      | 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/h211-B110 sandwich immunoassays. Analogs hCGβ-δ\(^{193}:3:100\)D and hCGβ-δ\(^{193}:100\)DA, which lack the tensor loop were detected in B111 assays indicating that their seatbelts are latched to βCys\(^{10}\) (not shown). The seatbelt of hCGβ-C93A,C108A is also known to be latched to βCys\(^{10}\) based on its ability to be recognized by B111 (Table I).

Constructs used in transfection

| α + hCGβ | 1.80 ± 0.05 |
| α + hCGβ-C93A,C108A | Not detected |
| α + hCGβ-C93A,C108A | 0.23 ± 0.01 |
| α + hCGβ-δ\(^{193}:100\)D | Not detected |
| α + hCGβ-δ\(^{193}:3:100\)D | 1.16 ± 0.29 |
| α + hCGβ-δ\(^{193}:100\)DA | Not detected |
| α + hCGβ-δ\(^{193}:100\)DA | 0.21 ± 0.09 |
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.

We suggest this is due to the disruption of the intersubunit disulfide caused by formation of the tensor disulfide. This suggests that the seatbelt latch disulfide forms after a significant amount of cross-link. These include: α-R55C, α-B42C, α-K45C, α-Q50C, α-Y88C, α-H90C, and α-S92C.

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 expected to stabilize the heterodimer. 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. 5a), but did not alter its recognition by conformation-dependent antibodies B101, B111, or B112 (Fig. 5b). 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. 5a) or its ability to be recognized by these antibodies (Fig. 5b). If these concentrations of BME had disrupted the tensor disulfide in the heterodimer, they would have rendered βCys93 and/or βCys100 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 βCys26 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 βCys110 can serve as seatbelt latch sites. Their abilities to compete with βCys26 as a latch site appears to vary inversely with their distance from βCys26, 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 βCys26.

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 βCys26. As shown (Table I), either tensor cysteine (i.e., βCys89 or βCys100) can compete with βCys26 for latching the seatbelt. Indeed, the proximity of the tensor cysteines to βCys110 makes it likely that the seatbelt would become latched to either βCys89 or βCys100 before it becomes latched to βCys26. 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 βCys89 or βCys100 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 βCys89 and βCys100 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 βCys110. 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 βCys26 and,

### Table VI

| Cross-linked heterodimer | Initial heterodimer | Amount after BME | Cross-linked after BME | Disulfide exchange |
|--------------------------|---------------------|------------------|------------------------|-------------------|
| 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                | 3.80             | 0.20                   | >95               |
| α-L48C + hCGβ-KDEL      | 4.01                | 3.95             | <0.1                   | 100               |

### Table VII

Cross-linked hFSH and hTSH heterodimers

| α-Subunit analog | Stability at acid pH | hFSHβ | hFSHβ-KDEL | TSHβ |
|-----------------|---------------------|-------|------------|------|
| 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 |      |
Redox Control of Human Glycoprotein Hormone Assembly

Key Roles in Heterodimer Assembly

Most human glycoprotein hormone heterodimers are assembled by a process in which a hormone heterodimer is 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 (7), may be crucial for heterodimer assembly 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 was readily secreted from cells and detected in assays employing B101 for capture and either 125I-B110 or 125I-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-893:100DA or an analog having the KDEL endoplasmic reticulum retention signal were co-expressed with the α-subunit.

**Fig. 5.** Influence of BME and IA treatment on hCG and the free β-subunit. Equimolar amounts of hCG and free β-subunit (10 × 10⁷ 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, 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.

**A** Low BME/IA Pretreatment of hCGβ Reduced its Ability to Combine with the α-Subunit

| Heterodimer Formed | β-Mercaptoethanol (mM) |
|--------------------|------------------------|
| 3                  | 0                      |
| 2                  | 0.17                   |
| 1                  | 0.5                    |
| 1                  | 2.0                    |

| hCGβ-Subunit       |
|--------------------|
| hCG                |

**B** Low BME/IA Pretreatment Did Not Affect the Seatbelt Latch Disulfide of hCG or hCGβ

| Ratio of B111/B112 | β-Mercaptoethanol (mM) |
|--------------------|------------------------|
| 1.25               | 0                      |
| 1.00               | 0.17                   |
| 0.75               | 0.5                    |
| 0.50               | 2.0                    |

| hCGβ              |
|--------------------|
| hCG                |

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. Restoration 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.

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4 hCGβ-893:100DA, 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 125I-B110 or 125I-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-893:100DA or an analog having the KDEL endoplasmic reticulum retention signal were co-expressed with the α-subunit.
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 β-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 βCys110 were latched to any other cysteine in the molecule. This showed that the end of the seatbelt was latched to βCys26, 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 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 NH2-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 αVal53, Ser55, Ser57 form hydrogen bonds with hCGβ-Asp99,Gly101 and possibly hCGβ-Thr97 (1, 2), a phenomenon that would constrain tensor cysteine hCGβ-Cys100 to a region nearby hCGβ-Cys93 (Fig. 6). In hFSH, these α-subunit residues form hydrogen bonds with hFSHβ-Asn93, Thr97 (3), thereby constraining tensor cysteine hFSHβ-Cys94 by hFSHβ-Cys97. 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-
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 βCys26 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 material 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.

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