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Biochemistry

Combination of rat lutropin subunits occurs early in the secretory pathway

(sulfation/pituitary hormones/biosynthesis/luteinizing hormone)

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ABSTRACT **The combination of lutropin (LH; luteinizing hormone) α and β subunits was examined in rat pituitaries incubated with [^{35}S]methionine or [^{35}S]sulfate. Combination was assessed by using antiserum directed against the β subunit. The data show that combination of most of the subunits proceeds rapidly, well before the addition of sulfate and prior to the processing of asparagine-linked oligosaccharides to the complex form. Thus, combination appears to initiate in the endoplasmic reticulum and does not require those post-translational modifications. We observed that two forms of the LH-α subunit were processed—one that is secreted into the medium not associated with the LH β subunit and another secreted as part of the α–β dimer. Both forms of the α subunit are sulfated, and the data suggest that subsequent to sulfate addition, secretion of free α subunit and the dimer occur independently by separate pathways.**

The pituitary hormones lutropin (LH), follitropin (FSH), and thyrotropin (TSH) are members of a family of structurally similar glycoproteins that includes the placental hormone, human choriogonadotropin (hCG). Each hormone is composed of two dissimilar, noncovalently-associated subunits designated α and β, which contain one or two asparagine-linked carbohydrate groups. Within a species, the amino acid sequences of the α subunits are identical, and the biological specificity of these hormones resides in their respective β subunits (1).

Although the chemical structures of the α and β subunits are well characterized, only recently has information concerning the synthesis and processing of the subunits emerged. The subunits are translated from separate mRNAs (2–4), and high mannose oligosaccharide units are transferred to asparagine residues in the nascent chains as they cross the endoplasmic reticulum (5). After release of the completed subunits from the ribosomes, these oligosaccharides are processed by enzymes localized within the Golgi complex. Recent studies have detected sulfate covalently linked to oligosaccharides on the α and β subunits of LH and thyrotropin but not of choriogonadotropin (6, 7). Thus, these hormones are subjected to several post-translational steps before they emerge from the cells.

A crucial step in the production of these hormones is the joining of the subunits. The timing and location of their combination after synthesis has not been established, and it is not known if maturation of the oligosaccharide side chain or sulfation (or both) is required for coupling of the subunits. We have demonstrated that, in the anterior lobe of the rat pituitary, LH and thyrotropin subunits could be labeled intracellularly with [^{35}S]sulfate and [^{35}S]methionine (7). During this work we observed that antisera directed against the bovine LH-β subunit precipitated the intact hormone. This observation permitted us to analyze the α–β subunit combination event in cells.

Here, we present evidence that the assembly of LH subunits occurs within the first few minutes of protein synthesis, well before the mature hormone is secreted into the medium. The combination event occurs before addition of complex sugars and sulfate.

MATERIALS AND METHODS

[^{35}S]Methionine and [^{35}S]sulfate (500–1,000 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels) were purchased from Amersham. Rabbit antiserum against reduced and carboxymethylated (RCM) LH-α subunit, native and RCM LH-β subunits, and intact LH were generously provided by J. Pierce. All antigens were purified from bovine pituitaries. These antisera have the following specificities: LH antiserum precipitates intact LH and free subunits:LH-β subunit antiserum precipitates intact LH and free β subunit; and antisera against RCM LH-α and RCM LH-β subunits precipitate only free α and β subunits, respectively. Endoglycosidase H was obtained from F. Maley (New York State Department of Health, Albany, NY).

Isolated anterior pituitaries from castrated male rats were preincubated in Krebs–Ringer bicarbonate buffer for 30 min and incubated in the same buffer with 50 μCi of [^{35}S]methionine or 240 μCi of [^{35}S]sulfate per ml for the indicated times, and the tissue was then homogenized in 1 ml of phosphate-buffered saline as described (7, 8). Immunoprecipitation was performed at 4°C for 18 hr in 20 mM Tris·HCl, pH 8.0/150 mM NaCl/5 mM EDTA/0.5% Triton X-100/0.1% $NaDodSO_4$.

Antigen–antibody complexes were precipitated with *Staphylococcus aureus* cells (Cowan strain), and the proteins were analyzed on $NaDodSO_4$/polyacrylamide gels as described (9). Under the conditions of $NaDodSO_4$/polyacrylamide gel electrophoresis used here, LH dissociates into the α and β subunits.

Subunit dissociation was performed as follows: 100-μl aliquots of the homogenate were adjusted to 0.1% $NaDodSO_4$ and 2% (vol/vol) 2-mercaptoethanol and were heated for 2 min at 100°C. The reactions were cooled, and the products were immunoprecipitated.

Labeled proteins were digested with endoglycosidase-H by the method of Bielinska *et al.* (10) except that solubilization was performed in the above immunoprecipitation buffer.

RESULTS

Anterior pituitary lobes of rats were incubated for 5 hr with [^{35}S]methionine, and the products were immunoprecipitated with LH subunit-specific antisera (Fig. 1). Antiserum directed against the RCM α subunit (lane 1) precipitated a protein (M_r, 22,000) that corresponded to the rat pituitary α subunit pre-

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Abbreviations: LH, lutropin (luteinizing hormone); RCM, reduced and carboxymethylated.

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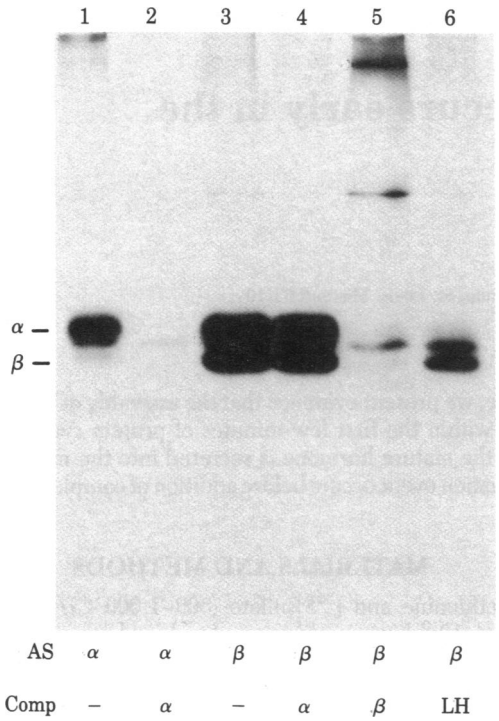

FIG. 1. Incorporation of [^{35}S]methionine by rat anterior pituitaries. Rat anterior pituitaries were incubated for 5.0 hr. The tissue was homogenized and intracellular protein was precipitated with anti-RCM α subunit (lanes 1, 2), or anti-native β subunit (lanes 3–6) antisera. "Comp" refers to competition with the addition of 20 μg of purified α subunit (lanes 2 and 4), of β subunit (lane 5), or of bovine LH (lane 6) to the immunoprecipitation reaction mixtures prior to the addition of antisera (AS). Under the conditions of $NaDodSO_4$/polyacrylamide electrophoresis used here, LH dissociates into its subunits.

viously described (7). Two products reacted with antiserum directed against the β subunit (lane 3), one of which comigrated with the α protein.

This result suggested that the anti-β subunit antiserum precipitated an α–β subunit complex by antibody recognition of determinants on the β subunit. However, our data could not exclude the presence of anti-α subunit antibodies in this antiserum. To distinguish between these possibilities, a series of competition experiments with purified LH and its subunits was performed (Fig. 1). If the suspected α protein was precipitated as a complex with the β subunit, both bands should be reduced or eliminated in the presence of unlabeled β subunit, whereas addition of purified α subunit should have no effect on the appearance of the two proteins. Unlabeled native β subunit, but not α subunit, prevented the appearance of both labeled proteins (Fig. 1, lanes 4 and 5). Unlabeled α subunit prevented the precipitation of labeled α subunit by anti-α subunit antiserum (Fig. 1, lane 2). When LH was added, none of the α subunit and much less of the β subunit was observed (Fig. 1, lane 6). The residual amount of β protein apparently was free subunit with which intact LH did not compete. The band that migrates between the α and β proteins is a nonspecific contaminant (Fig. 1, lanes 2, 5, and 6); it is observed when nonimmune serum supplants subunit-specific antiserum, and purified subunits or the intact subunit did not compete with it.

Further evidence that LH was precipitated by anti-LH-β subunit antiserum was obtained by dissociating the dimer before addition of antisera. If the combined LH reacted with anti-β subunit antiserum, then dissociation of LH into subunits prior to immunoprecipitation should eliminate the appearance of the α subunit. Accordingly, reaction mixtures containing labeled proteins accumulated in the tissue or medium were heated for 2 min at 100°C in the presence of 2% 2-mercaptoethanol and 0.1% $NaDodSO_4$, and the proteins were precipitated with antiserum against α or β subunits. Under these conditions, only one protein was precipitated with anti-β subunit antisera (Fig. 2, lanes 4 and 8). It was also evident that only a fraction of LH was secreted into the medium (compare lanes 7 and 3 in Fig. 2; see below). Taken together, this and the previous experiments demonstrate that intact LH was precipitated by anti-LH-β subunit antiserum.

We next wanted to determine the temporal sequence between combination and maturation of the asparagine-linked oligosaccharides. To investigate this point, anterior pituitary lobes were incubated for 15, 45, and 90 min in the presence of [^{35}S]methionine. The intracellular labeled proteins were immunoprecipitated with antiserum against LH-α subunit, LH-β subunit, or LH, and the products then were digested with endoglycosidase H (Fig. 3). This enzyme cleaves the di-*N*-acetylchitobiose bond of high mannose oligosaccharides but is ineffective on complex carbohydrate structures (11). From 15 to 90 min of incubation, both components precipitated by anti-LH-β subunit antiserum were sensitive to endoglycosidase H (Fig. 3, lanes 4, 10, and 16). It was also apparent that the deglycosylated forms of α and β subunits migrate at the same position on the gel (Fig. 3, arrow). This was expected because the molecular weights of the corresponding aglycoproteins are very similar (1). By 90 min, significant endoglycosidase H resistance of the α subunit of the dimer was observed (Fig. 3, lane 16). It was apparent that the α subunit precipitated by anti-subunit α antiserum (free α-subunit) was completely sensitive to endoglycosidase H at all times (Fig. 3, lanes 2, 8, and 14). This is consistent with the findings of Ruddon *et al.* (12) that, in cultured choriocarcinoma cells, the free α subunit remains endoglycosidase H sensitive after 2 hr of incubation. Some of the α subunit precipitated by anti-LH antiserum (which includes α subunit

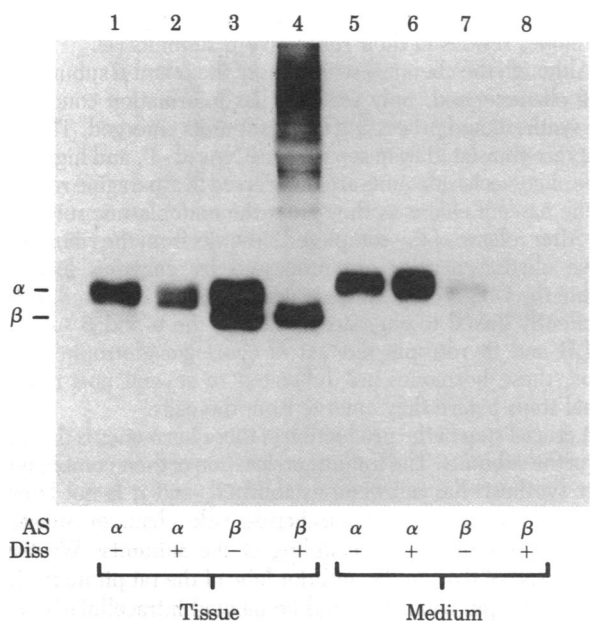

FIG. 2. Dissociation of labeled LH in the tissue and medium. Anterior pituitary tissue was incubated with [^{35}S]methionine for 6 hr, and the tissue extract was immunoprecipitated (lanes 1–4). The medium (lanes 5–8) was collected and also immunoprecipitated. Prior to immunoprecipitation, half of each of the tissue and medium extracts was boiled at 100°C for 2 min in the presence of 2% 2-mercaptoethanol and 0.1% $NaDodSO_4$ to dissociate LH dimers into its component subunits. AS, antiserum; Diss, dissociation.

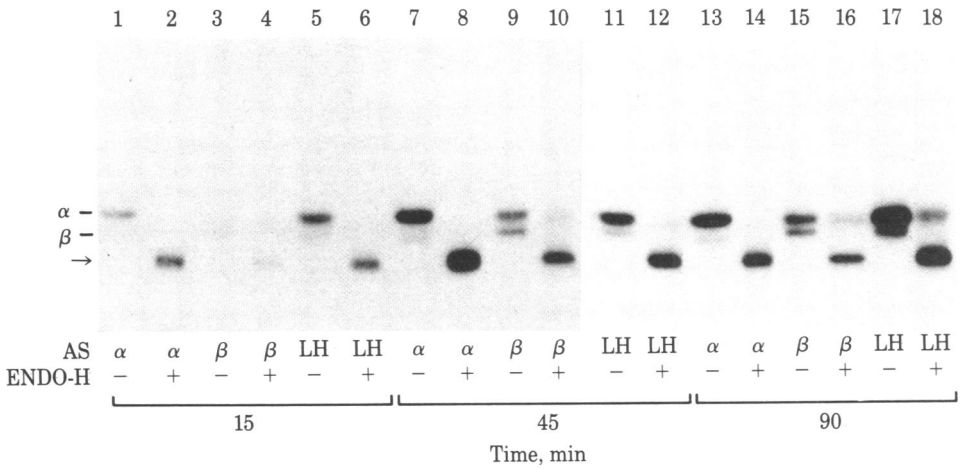

FIG. 3. Endoglycosidase H sensitivity of the LH dimer. Anterior pituitaries were incubated with [^{35}S]methionine for 15, 45, or 90 min and homogenized as described. The immunoprecipitated products were solubilized, denatured, and treated with 0.001 units of endoglycosidase H (Endo-H) according to Bielinska *et al.* (10). AS, antiserum.

combined in LH) was resistant (compare lanes 14 and 18 in Fig. 3). Thus, free α subunit did not form complex oligosaccharides in this interval, whereas α subunit in the α–β dimer did. These results show that the combination event precedes the formation of endoglycosidase H-resistant material—i.e., complex sugar addition.

The previous experiments suggested that the combination event occurs early in the secretory pathway. Therefore, we studied the time course of the α–β combination to determine the earliest time when the dimer can be detected (Fig. 4). Pituitaries were incubated for 3–10 min, and the proteins were immunoprecipitated with antisera against the β and α subunits.

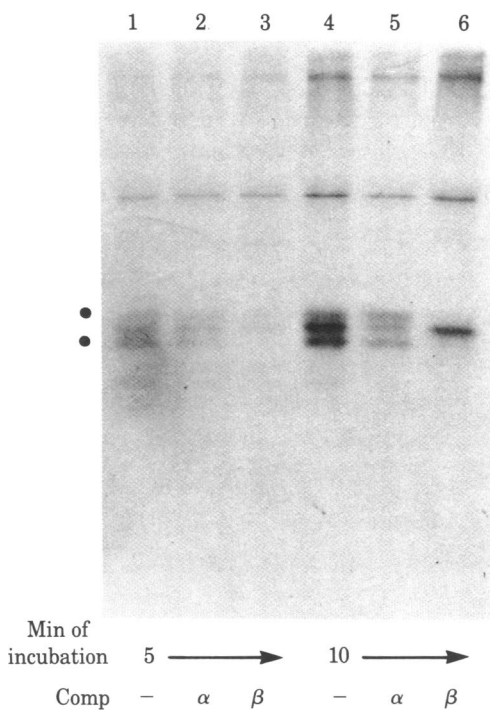

FIG. 4. Short-term incubations of pituitary fragments. Anterior pituitaries were incubated for 5 or 10 min with [^{35}S]methionine, and the tissue extract was immunoprecipitated by anti-native β subunit antiserum. Competition (Comp) with 20 μg of purified RCM LH subunits was performed as described in the legend of Fig. 1. The dots denote the α/β subunits.

At 3 min of incubation, no detectable labeled proteins were observed (data not shown), but after 5 min and especially after 10 min of incubation, LH was seen. Competition with the immunoprecipitated combined LH form was caused by addition of purified β subunit but not addition of α subunit (Fig. 4, lanes 2 and 3). The nonspecific middle band was more prevalent during these short incubation periods because the incorporation of label into the subunits was less. Therefore, at the earliest time point that labeled subunits were detectable, the coupled form of the hormone was present. This implies that combination initiates in the endoplasmic reticulum soon after the subunits are synthesized.

The above studies were performed with [^{35}S]methionine, which measures *de novo* synthesis of the subunits. Previously, we had observed that cycloheximide blocked the incorporation of methionine but not of [^{35}S]sulfate into the subunits (7). Moreover, sulfate is linked to the nonreducing termini of the LH oligosaccharide side chains (6). These observations imply that sulfation is one of the final events in the maturation of the subunits and occurs after the joining of subunits. To examine this point further, pituitaries were incubated with [^{35}S]sulfate, and the intracellular proteins were immunoprecipitated (Fig. 5, lanes 1 and 2). In the presence of anti-LH-β subunit or anti-LH antiserum, only the dimer was precipitated. No free α subunit labeled with sulfate could be detected in the tissue with anti-α subunit antiserum (data not shown), contrasting with the methionine labeling data that showed free α subunit was present (see Fig. 1, lane 1). In the medium, however, a large excess of free sulfate-labeled α subunit was observed (Fig. 5, lane 4). It was also clear that there was a slight difference in the mobilities of the free α subunit in the medium and the α subunit derived from combined LH (Fig. 5, lanes 3 and 4; see *Discussion*)—the free α subunit migrated more slowly. Moreover, in the medium, only a fraction of the total sulfate-labeled immunoprecipitable material was LH (compare lanes 1 and 3 in Fig. 5). A similar difference was obtained with methionine-labeled proteins (Fig. 2, lanes 3 and 7). These data are consistent with other observations that show a preponderance of α subunits in media containing choriocarcinoma cells (13) and mouse pituitary cells (14).

We also used antiserum directed against RCM LH-β subunit to assess the presence of free β subunit in the tissue. Antiserum directed against reduced denatured subunit should precipitate free β subunits that have not yet achieved a native configuration and subsequent combination with α subunit. The RCM β sub-

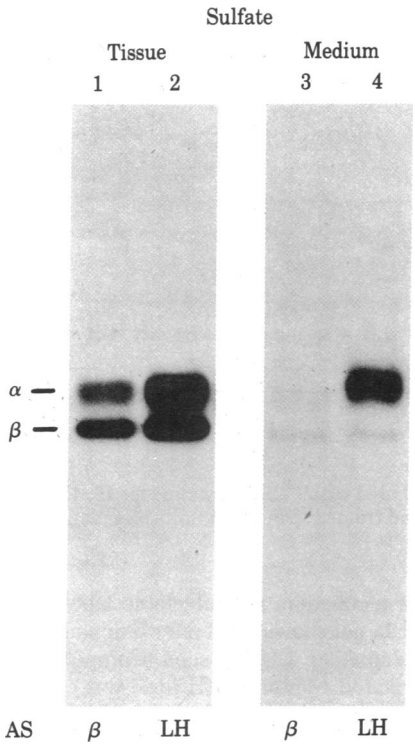

FIG. 5. Sulfate labeling of LH. Anterior pituitaries isolated from ovariectomized animals were incubated for 6 hr with [^{35}S]sulfate. Proteins in the tissue and medium were precipitated with anti-LH-β subunit and anti-LH antisera (AS).

unit antiserum precipitated some free β subunit labeled with methionine, but none was observed in pituitaries labeled with sulfate (data not shown). Because sulfate is attached to complex sugars of LH oligosaccharides and only the combined molecule contains sulfate, the data show that sulfation can occur after the subunits are joined. The data further indicate that sulfation is not obligatory for subunit coupling.

If sulfate addition is a late reaction, then sulfate-labeled subunits should precede methionine-labeled protein into the medium. Accordingly, we performed a time course experiment with [^{35}S]methionine and [^{35}S]sulfate (Fig. 6). LH subunits (immunoprecipitated with anti-β subunit antiserum) in the tissue were sulfated within 5 min of incubation, and the LH appeared in the medium at 15–30 min (Fig. 6*B*). However, with methionine, at least 2 hr lapsed before labeled LH was detected in the medium. Free α subunit labeled with methionine required an intermediate length of time of about 1 hr before it was secreted (Fig. 6*A*, lanes 2 and 4).

DISCUSSION

It has been suggested that the coupling of the thyrotropin α–β pair is one of the final events in the secretion of the intact hormone (14). However, the data presented here demonstrate that the lutropin subunits combine early in the secretory pathway, well before the intact hormone is secreted. In fact, LH was detected at the earliest time point when labeled immunoprecipitable proteins could be visualized. We infer from these results that coupling initiates in the endoplasmic reticulum. Combination of hCG subunits at an early, endoglycosidase H-sensitive stage has been observed in chariocarcinoma cells (R. Ruddon, personal communication). Combination of the free β subunit to α subunit is completed in the endoplasmic reticulum and precedes sulfate addition. This conclusion is based on the lack of accumulated free sulfated subunits in the tissue. The data show that asparagine-linked oligosaccharides on LH mature to endoglycosidase H-resistant glycose units after the α and β subunits are joined. The penultimate reactions leading to endoglycosidase H resistance are associated with the Golgi (11, 15, 16). Thus, coupling is not dependent on the presence of fully mature oligosaccharide units, and LH serves as the substrate for the glycosyl- and sulfur-transferases in the Golgi apparatus or secretory granules.

The origin of the free, unassociated α subunit in the medium is not clear. Based on preliminary pulse–chase experiments, it is not derived from uncoupling of LH. If dissociation were the source, we might have expected to detect free β subunits in the medium which, in fact, were not observed. However, it is pos-

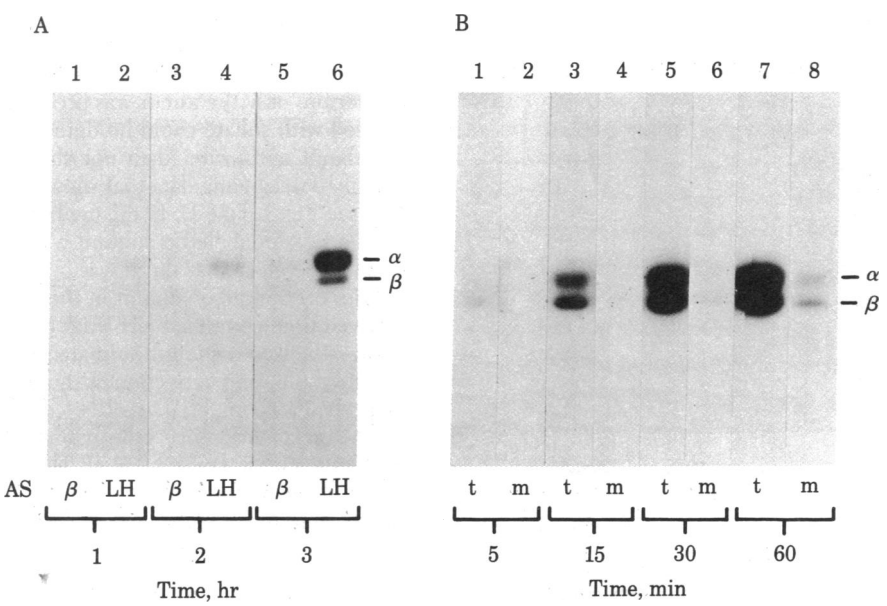

FIG. 6. Time course of methionine- and sulfate-labeled LH proteins. Rat anterior pituitaries from castrated rats were incubated with [^{35}S]methionine (*A*) or [^{35}S]sulfate (*B*) for the indicated times. In *A*, only the appearance of labeled proteins in the medium was assessed; AS denotes the antiserum used. In *B*, anti-β subunit antiserum was used to precipitate protein in the tissue (t) and media (m).

sible that uncoupled β subunits are preferentially degraded. Chin *et al.* (14) have suggested that accumulation of excess α subunits in a murine thyrotropin tumor was due to an enhanced intracellular degradation of the β subunits. Enhanced degradation of LH may explain also the small amount of LH elaborated into the medium. Another possibility for the low efficiency of LH release is that an essential component is absent or limiting (e.g., LH-releasing hormone) as a result of the *in vitro* conditions used here. Although our data do not assess these points directly, it is apparent that the impaired step limiting the appearance of LH in the medium occurs after sulfate attachment.

The pool of free α subunit appearing in the medium may represent a unique form not derived from the combined molecule. Several investigators have demonstrated the existence of two secreted forms of the α subunit in gonadotropin-producing cells: one that is associated with the native hormone and a free form that cannot associate with the β subunit. This free form has altered elution characteristics on gel filtration columns and on $NaDodSO_4$ gels (14, 17–21), in which it migrates slightly slower than the combined α subunit. Parsons *et al.* (20) have reported that the free α form in bovine pituitary contains some *O*-linked carbohydrate. In our experiments, the free α subunit form in the medium migrates slower than α subunit combined in LH. That the α subunit precipitated from tissue with anti-α subunit antiserum was sensitive to endoglycosidase H, whereas some of the α subunit precipitated with anti-LH antiserum was resistant to the enzyme, further emphasizes the difference in the kinetics of processing between the combined and free forms. Because very little of the altered form of the α subunit accumulates in the cell, modification of free α subunits may be a final step in secretion, occurring at the plasma membrane or the extracellular space of the pituitary. Based on this observation and the structural and kinetic differences in the processing of combined and free α subunits, it is suggested that the two forms of α subunit follow different secretory pathways. Evidence for the existence of two secretory pathways involving mature corticotropin and a viral glycoprotein in the pituitary cell line AtT-20 has been reported (22). Alternatively both the free subunit and LH may traverse the same pathway but at different rates. Whether separate or common pathways are used, it is clear that the cell distinguishes free α subunits from those complexed with β subunits.

The physiologic role of the free α subunit is unclear. It may represent a separate biologic entity that does not require the β subunit for activity. Modification of the subunit may be a mechanism for eliminating free α subunits that might interfere with the secretion of the intact dimer. Thus, both forms of the α subunit are derived from a common pool of α subunits but, ultimately, the quantity of free α subunit appearing in the medium is determined by the availability of β subunits.

Note Added in Proof. During preparation of this manuscript, a paper appeared showing that combination of the thyrotropin subunits also initiates in the endoplasmic reticulum (23).

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