Metabolic labeling of lutropin with [³⁵S]sulfate

(gonadotropins/pituitary/sulfation of secretory proteins)

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ABSTRACT Chemical analyses have previously detected sulfate linked to the oligosaccharides of lutropin isolated from bovine and human pituitaries. To determine whether lutropin could be metabolically labeled with sulfate, isolated bovine and rat pituitaries were incubated with [³⁵S]sulfate. In both species, two major labeled products were immunoprecipitated with antisera specific to lutropin subunits. Incorporation into the subunits occurred posttranslationally since it was not blocked by cycloheximide, which did, however, block the incorporation of radiolabeled methionine. Metabolic labeling with [³⁵S]sulfate provides a valuable approach for examining the biosynthetic processing of lutropin and the physiological role of sulfate in this hormone.

The glycoprotein hormones lutropin (LH), thyrotropin, follicle-stimulating hormone, and human chorionic gonadotropin (hCG) consist of two noncovalently joined polypeptide chains, the α and β subunits. Within a species, the amino acid sequences of the α subunits of these hormones are nearly identical (1) and, in humans, only a single α subunit gene has been detected (2). Biological specificity of these hormones is conferred by differences in their β subunits although both subunits are required for activity (3). The asparagine-linked oligosaccharides of the pituitary hormones and the placental hormone, hCG, differ in composition (4–7) and in resistance to glycosidases (8). Chemical analysis by Parsons and Pierce (8) recently revealed a further structural difference; sulfate is linked to the nonreducing termini of oligosaccharides of bovine and human LH but not of hCG. The structure of the oligosaccharide chains is important because it affects the circulating half-life and, thus, the activity of some glycoproteins (9, 10). Also, because the peptide chains of placental and pituitary α subunits are essentially identical, comparison of their oligosaccharide chains provides an approach for studying the tissue specificity of oligosaccharide chain maturation. This paper reports an investigation of the intracellular attachment of [³⁵S]sulfate to LH in isolated rat pituitaries and bovine pituitary slices. We show that isolated pituitaries incorporate [³⁵S]sulfate into rat and bovine LH. Labeling with sulfate should provide a useful new approach to examine the synthesis and maturation of pituitary glycoprotein hormones.

MATERIALS AND METHODS

[³⁵S]Sulfate (carrier-free) and [³⁵S]methionine (500–1000 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) were from Amersham. Antisera against LH and against reduced carboxymethylated subunits were as described (11).

Fresh bovine pituitaries were obtained from a local slaughterhouse and kept in ice-cold Krebs–Ringer bicarbonate buffer

(with MgCl₂ substituted for MgSO₄)/0.2% glucose/1% bovine serum albumin containing penicillin G at 100 units per ml. A Stadie–Riggs tissue slicer was used to divide the tissue into segments 0.5 mm thick. Slices weighing ≈ 10 mg were incubated in 95% O₂/5% CO₂ at 37°C in 0.5 ml of the same bicarbonate buffer except that bovine serum albumin was replaced by soybean trypsin inhibitor at 100 μ g/ml. After 15 min of incubation of the tissue slices, 0.5 ml of medium containing [³⁵S]sulfate (100 μ Ci/ml) or [³⁵S]methionine (50 μ Ci/ml) was added. Isolated rat hemipituitaries from castrated or noncastrated animals were incubated in the same manner. The tissues were then homogenized, and protein was precipitated with trichloroacetic acid or by antisera and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis as described (12). Labeled peptides were detected by fluorography (13) of the gels. Immunoprecipitations used a double-antibody technique with samples in physiological saline/1% Triton X-100/1% sodium deoxycholate. Reduction and carboxymethylation of LH was carried out in 8 M urea as described by Hirs (14). Salts and urea were then removed by gel filtration through Sephadex G-25. Treatment with hydrazine was as described (15) except that its duration was 10 hr rather than 5.5.

RESULTS

Slices of steer anterior pituitary tissue incubated for 3 hr with [³⁵S]sulfate incorporated the label into several products (Fig. 1A, lane 1). In addition to high molecular weight products near the top of the gel, there were prominent labeled products of $\approx 22,000$ and $25,000$ daltons. These proteins were precipitated by antiserum to LH (lane 2) but not by nonimmune serum (lane 3).

Products released into the medium were also examined (Fig. 1B, lane 1). Only one labeled protein appeared in the 20,000- to 25,000-dalton region of the gel, and its mobility was between those of the two LH-related products from the tissue. This protein was precipitated by antiserum directed against either native LH (lane 2) or against reduced carboxymethylated α subunit (lane 3). These results suggest that the labeled product in the medium was free α subunit because the antiserum against reduced carboxymethylated α subunit crossreacts poorly with intact LH (11). Antiserum against LH is capable of binding either free or combined subunits.

The number of products labeled by [³⁵S]methionine (lane 6) was much greater than that of products labeled by sulfate (lanes 1 and 5). It is striking, for example, that no detectable sulfate-labeled material comigrated with growth hormone which, in the presence of [³⁵S]methionine, was the major labeled product. Thus, probably only a few pituitary proteins are sulfated.

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Abbreviations: LH, lutropin; hCG, human chorionic gonadotropin.

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FIG. 1. (A) Incorporation of [^{35}S]sulfate by bovine pituitary slices. Bovine pituitary slices were incubated for 3 hr in medium containing [^{35}S]sulfate. The tissue was homogenized and protein was precipitated with trichloroacetic acid (lane 1), rabbit anti-bovine LH serum (lane 2), or nonimmune rabbit serum (NRS, lane 3). Products were analyzed by NaDodSO₄/polyacrylamide gel electrophoresis and subsequent fluorography. (B) [^{35}S]Sulfate-labeled products in the medium. Medium was collected after incubation of pituitary slices as in A. Labeled products were precipitated with trichloroacetic acid (lane 1), antiserum against bovine LH (lane 2), antiserum against reduced carboxymethylated LH α subunit (lane 3), or nonimmune rabbit serum (lane 4). Total products in tissue labeled with [^{35}S]sulfate or [^{35}S]methionine (lanes 5 and 6) were precipitated with trichloroacetic acid. GH, growth hormone.

The terminal location of sulfate on the oligosaccharides of LH (8) suggests that its attachment is one of the final events in the maturation of the oligosaccharide. Consequently, sulfate labeling of LH should continue for some time even if protein synthesis is blocked. Cycloheximide, which completely inhibited the incorporation of [^{35}S]methionine into LH (Fig. 2, lane 2), did not prevent the labeling of LH with [^{35}S]sulfate (lane 4). Material released into the medium was also examined and

FIG. 2. Effect of cycloheximide on methionine and sulfate incorporation. Bovine pituitary slices were incubated 3 hr in medium containing [^{35}S]methionine (lanes 1, 2, 5, and 6) or [^{35}S]sulfate (lanes 3, 4, 7, and 8) with (lanes 2, 4, 6, and 8) and without (lanes 1, 3, 5, and 7) 1 mM cycloheximide. Products from tissue (lanes 1–4) and medium (lanes 5–8) were precipitated with trichloroacetic acid and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis.

FIG. 3. Immunoprecipitation by LH subunit-specific antisera. After the incubation of bovine pituitary slices with [^{35}S]sulfate, labeled protein was precipitated from the tissue homogenate with 50% saturated ammonium sulfate and then reduced and carboxymethylated. Lanes 1–3, products precipitated by nonimmune serum and by antisera against reduced carboxymethylated LH β and α subunits, respectively; 4, total reduced carboxymethylated products before immunoprecipitation.

showed similar results (lanes 5–8). These data show that sulfate is attached to LH posttranslationally and that the secreted LH still contains the [^{35}S]sulfate label. Cycloheximide decreased the labeling of the high molecular weight products, suggesting that their sulfation is more closely coupled to synthesis than is that of LH.

Previously, sulfate attached to LH was detected directly by chemical analysis of intact LH and isolated α subunits (8). Its presence in β subunits was deduced by the difference in the values for α and intact LH. The occurrence of sulfate-labeled products that migrated in positions similar to those of LH subunits and were immunoprecipitated by LH antiserum suggested that both subunits were labeled. Strong evidence for sulfate labeling of the β subunit was obtained by immunoprecipitating dissociated LH subunits with antisera specific to each subunit (Fig. 3). After the subunits were dissociated by reduction and carboxymethylation, two distinct proteins were precipitated with subunit-specific antisera (lanes 2 and 3). Non-immune serum did not precipitate any products (lane 1).

In addition to the oligosaccharide,[†] potential sites for the linkage of sulfate are tyrosine residues in the peptide chain. Sulfation of tyrosine residues has been detected in a number of proteins [e.g., fibrinogen (17) and gastrin (18)]. LH purified by gel filtration was used to examine whether the sulfate was

[†] Treatment of either unlabeled LH or the tryptic glycopeptides of unlabeled LH α subunit with arylsulfatase A or B failed to release the covalently bound sulfate (unpublished observations). However, human liver arylsulfatase B removes the sulfate from both the *N*-acetylgalactosaminyl fragments and the *N*-acetylglucosylaminyl fragments resulting from Smith degradation (16), indicating that sulfate is probably linked to the C-4 hydroxyl of the *N*-acetylhexosamines.

linked to the peptides or oligosaccharides of LH. Oligosaccharide chains were released from sulfate-labeled LH by treatment with hydrazine. This treatment should completely degrade the peptide but not the oligosaccharide moieties. After such treatment of labeled LH isolated from the tissue, [35 S]sulfate remained attached to material large enough to elute in the excluded volume of a Sephadex G-15 column (Fig. 4B), suggesting that the label was attached to an oligosaccharide. The elution profile of free [35 S]sulfate is shown in Fig. 4A. Material in the excluded fractions from the Sephadex G-15 column was analyzed further on a concanavalin A-Sepharose column (Fig. 5A). A portion of the label bound specifically to the column, as shown by elution with the ligand α -methylmannoside but not by D-galactose, which does not bind to this lectin. Of the label applied, 16% was eluted with α -methylmannoside and 5% remained bound to the column. Sulfate-labeled LH from the medium (mainly α subunit) was analyzed similarly (Fig. 5B) after digestion with Pronase rather than with hydrazine. In this case, 24% of the input radioactivity was eluted specifically by α -methylmannoside. These results show that at least a portion of the label is linked to oligosaccharide. Virtually all LH molecules bind to concanavalin A (19, 20), but it is not known whether all three of the oligosaccharide chains interact with the lectin. The oligosaccharides of LH may be heterogeneous; the hormone can be separated into two fractions with different affinities for concanavalin A (19).

FIG. 4. Gel filtration of sulfate-labeled LH after treatment with hydrazine. [35 S]Sulfate- (A) or hydrazine-treated (B) sulfate-labeled LH was analyzed on a 1×19 cm Sephadex G-15 column eluted with 10% ammonium acetate. Use of a high ionic strength buffer was necessary to minimize charge exclusion of material from the Sephadex. At low ionic strength, ions with high charge density (e.g., free sulfate) eluted in the void volume. One-eighth of each fraction in B was assayed. The remainder of fractions 13–16 was pooled and analyzed further (see Fig. 5A). Elution positions of blue dextran (the void volume) and of [3 H]mannose (Man) are indicated. Sulfate-labeled LH for this analysis was prepared by gel filtration of a labeled tissue homogenate on a 2×90 cm Sephadex G-100 column eluted with 1% ammonium bicarbonate.

FIG. 5. Concanavalin A-Sepharose chromatography. (A) Sulfate-labeled LH was hydrolyzed by treatment with hydrazine, and the product was eluted in the excluded volume of a Sephadex G-15 column (Fig. 4B, fractions 13–16) was analyzed on a 1-ml concanavalin A-Sepharose column in 120 mM NaCl/30 mM Tris-HCl, pH 7.5/1 mM $MgCl_2$ /1 mM $MnCl_2$ /1 mM $CaCl_2$ /0.1% Triton X-100. Each fraction represents 1 column vol. After 20 column vol, 0.2 M D-galactose (D-Gal) was added to the eluant and, after 30 column vol, 0.2 M α -methyl-D-mannoside (d-MeMan) was added. (B) Sulfate-labeled LH isolated from the medium by gel filtration on Sephadex G-100 was reduced and carboxymethylated and the product was then digested at 37°C with two $\times 100$ - μ g portions of Pronase for 48 hr in 1 ml of 0.1 M ammonium bicarbonate/5% ethanol. Less than 10% of the label remained precipitable by trichloroacetic acid. The Pronase digest was boiled for 5 min and analyzed as in A.

To examine whether sulfate labeling of gonadotropins is restricted to the bovine species, we labeled products of anterior pituitaries from castrate rats (Fig. 6). Sulfate was incorporated into two products (lane 4) that migrated in positions similar to the labeled subunits of bovine LH. These products were immunoprecipitated by antiserum to bovine LH (lane 5). Similar methionine-labeled proteins were immunoprecipitated (lane 2), but they represented fairly minor components among the total methionine-labeled products (lane 3). The most prominent methionine-labeled protein is growth hormone (12). No methionine or sulfate-labeled material was precipitated by nonimmune serum (lanes 1 and 6). In the medium, we observed only a single LH-related product labeled with methionine (lane 8) or with sulfate (lane 11). Thus, in rat pituitaries as in bovine pituitaries, predominately a single subunit, probably the α subunit, appeared to be secreted. Further evidence that we had correctly identified the two sulfate-labeled products as LH was provided by comparison of sulfate incorporation by pituitaries from non-castrate and castrate animals (Fig. 7). Much more label was incorporated into the two products by pituitaries from the castrate rats. This correlates well with the increased synthesis of LH in castrate animals, which no longer have gonadal feedback inhibition of gonadotropin biosynthesis (21). With longer exposure of gels, some labeling of LH was also observed in noncastrate

FIG. 6. Analysis of rat pituitary products. (A) Tissue. (B) Medium. Pituitaries were isolated from two castrate male rats. Half of each pituitary was incubated 6 hr in buffer containing [35 S]sulfate (lanes 4–6). Contralateral half pituitaries were incubated with [35 S]methionine (lanes 1–3). The pituitary tissue was homogenized and aliquots of the homogenate were immunoprecipitated by nonimmune rabbit serum (lanes 1 and 6) or rabbit anti-bovine LH serum (lanes 2 and 5) or were precipitated by trichloroacetic acid (lanes 3 and 4). One eighth of each precipitate was analyzed by NaDodSO₄/polyacrylamide gel electrophoresis except lane 3, which contained only 2% of the sample.

animals, indicating that sulfation of LH occurs in normal as well as in castrate animals.

DISCUSSION

Our results showing covalent linkage of sulfate to both subunits of bovine LH confirm the earlier analytical study (8). In addition,

FIG. 7. Products of castrate and noncastrate rats. Pituitaries were isolated from noncastrate male (lanes 1 and 4), castrate male (lanes 2 and 5), and castrate female (lanes 3 and 6) rats. Half of each pituitary was incubated 5 hr in medium containing [35 S]sulfate (lanes 1–3) and the other half was incubated with [35 S]methionine (lanes 4–6). Proteins in the tissue were precipitated with trichloroacetic acid and analyzed by NaDodSO₄/polyacrylamide gel electrophoresis and fluorography.

sulfate is detected in human LH (8), and we observed sulfation of rat LH, so that this modification of LH appears to have a widespread distribution among species. We provide evidence that some of the sulfate label is linked to the sugar of LH, but we cannot conclude that all of the incorporated LH [35 S]sulfate is linked to the oligosaccharides. The analyses for sulfate by chemical methods and by metabolic labeling provide complementary data. Chemical analysis assesses the steady-state level of sulfation of LH isolated from a large number of animals, while labeling with sulfate permits study of the biosynthetic process in individual pituitaries and of the role of sulfation in the maturation and turnover of LH.

Metabolic labeling with sulfate also provides an approach for examining the secreted form of LH. Analyses of the structure of LH have relied on material isolated from pituitaries. The structure of the circulating hormone has not been examined although differences have been observed between the oligosaccharides on intracellular and extracellular forms of glycoprotein hormones such as hCG (22, 23) and rat thyrotropin (24). In this study, we have compared the electrophoretic mobilities of the labeled LH α subunit from tissue and from the medium and detected a small difference. We do not know whether this change occurs intracellularly or during sample preparation. An additional consideration is that we observed mainly free α subunit in the medium; the free subunit may be processed differently than subunit combined in LH. We should be able to examine the secreted form of intact LH by stimulating its secretion with LH-releasing hormone.

Establishing whether sulfation of free α subunits can occur is of interest because many tumors and cell lines synthesize the gonadotropin α subunit. There is only one gene and, thus, one peptide sequence for the α subunit (2), but our results suggest the possibility of classifying α -subunit synthesis into two types, either placental or pituitary, according to whether sulfate is incorporated into the subunit.

The proposed structure of the oligosaccharides of LH is unique in two respects, the linkage of sulfates to the 4-position of terminal sugars (8) and the position of *N*-acetylglucosamine (7, 8). The characteristic structure of LH poses two major questions: (i) What factors determine that LH has this particular oligosaccharide structure? (ii) What is the physiological role of sulfate? With regard to the first question, there are two possibilities. Processing of LH is due to either recognition of specific structural regions in the hormone or a unique property of luteotrophs to modify glycoproteins. Several observations support the premise that pituitary cells have a general capacity for sulfation of glycoproteins. Autoradiography of pituitary cells pulse labeled with [35 S]sulfate detects incorporation by all cell types (25). Previously, this has been ascribed in large part to incorporation of sulfate into glycosaminoglycans (25, 26), but at least two pituitary glycoproteins besides LH contain sulfate. Bovine thyrotropin is sulfated (8), and we have detected sulfation of the corticotropin (ACTH) precursor, pro-opiomelanocortin, in rat pituitaries (unpublished data). These results suggest that, although attachment of sulfate to asparagine-linked oligosaccharides has been detected in only a few other instances (27, 28), sulfation of glycoproteins may occur commonly in the pituitary.

With regard to understanding the physiological significance of the sulfation of LH, comparison of LH and hCG is helpful. In addition to being structurally homologous, these two hormones also bind to the same receptors and have similar physiological effects (29). Because hCG lacks sulfate, this comparison eliminates the sulfate moieties as essential components in binding to receptors and in receptor-effector coupling. hCG con-

tains sialic acid at the nonreducing termini of its oligosaccharides, in positions analogous to sulfate in LH. Sialic acid and sulfate possibly serve the same function (8). In addition to their analogous location, both functional groups confer negative charge to their respective oligosaccharides, which otherwise are neutral. Removal of sialic acid from hCG does not markedly affect binding of the hormone to receptors (29), but the *in vivo* activity of the hormone is greatly diminished because it is rapidly cleared from circulation (30, 31). Thus, sialic acid appears to have an important role in slowing the clearance of hCG. Many similar instances have been observed in which nonreducing terminal sugars affect the clearance of their parent glycoproteins (for review, see ref. 9, 10). Glycoproteins with terminal *N*-acetylglucosamine, as in the proposed structure of bovine LH, often have very short plasma half-lives (9). This suggests that a function of sulfate groups, like sialic acids, is to decrease the rate of clearance of LH by blocking the terminal sugars. Sulfate-labeled LH should aid in finding chemical or enzymatic methods to remove sulfate from the hormone, so that direct analysis of the effects of this moiety on the clearance of LH can be made. Also, sulfate-labeled LH may permit examination of whether there is any loss of sulfate from the hormone during circulation similar to the loss of sialic acid from hCG.

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