The Occurrence of O-Acetylation during Biotinylation of Gonadotropin-releasing Hormone and Analogos

EVIDENCE FOR A REACTIVE SERINE*

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Brian T. Miller†, Thomas J. Collins‡, Gregg T. Nagle§, and Alexander Kurosky¶

From the †Department of Anatomy and Neurosciences, the ‡Marine Biomedical Institute, and the ¶Department of Human Biological Chemistry and Genetics, the University of Texas Medical Branch, Galveston, Texas 77550

Gonadotropin-releasing hormone (GnRH) and two of its analogs ([D-Lys]GnRH and des-Gly\textsuperscript{10}[-d-Trp]\textsuperscript{10}]-GnRH) were reacted with sulfonated N-hydroxysuccinimide esters of biotin that have been reported to react specifically with primary amino groups. Fractionation by reversed-phase high performance liquid chromatography demonstrated the occurrence of multiple biotinylated derivatives for each reacted peptide. These results were unexpected since GnRH and des-Gly\textsuperscript{10}[-d-Trp]\textsuperscript{10}]-GnRH contained no reactive amino groups and [D-Lys]\textsuperscript{10}GnRH had only one. Reaction of the biotinylated derivatives with hydroxylamine indicated that significant O-biotinylation had occurred. Mass spectrometric analyses established the stoichiometry of biotinylation and confirmed that substantial O-biotinylation of residue Ser\textsuperscript{4}, and to a minor extent Tyr\textsuperscript{3}, of GnRH and the two analogs had occurred. In contrast, the biotinylation of selected peptides unrelated to GnRH under identical reaction conditions indicated no significant evidence of O-acetylation of seryl residues. Strikingly, biotinylation of GnRH under denaturing conditions largely abolished O-acetylation, indicating that the observed O-biotinylation was dependent on peptide conformation. All the O-biotinylated derivatives displayed significantly reduced bioactivity. Taken together, these results give strong evidence that the Ser\textsuperscript{4} hydroxyl of GnRH has a significantly elevated intrinsic reactivity, which raises new questions concerning its putative role in the conformation and mode of action of the hormone. These results also demonstrate for the first time that the N-hydroxysuccinimide-biotin esters are capable of significant O-acetylation and may be generally useful reagents for detecting highly reactive hydroxyamino acid residues.

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†To whom correspondence should be addressed: Dept. of Human Biological Chemistry and Genetics, the University of Texas Medical Branch, Galveston, TX 77550. Tel: 409-772-2771; Fax: 409-772-4965.

GnRH\textsuperscript{*} is a linear decapeptide produced by neurosecretory cells in the anterior hypothalamus and in extrahypothalamic neuronal systems of several species (Sherwood, 1987). Released in discrete pulses from hypothalamic nerve terminals, GnRH is transported to the anterior pituitary, where it effects the release of LH and FSH (Fink, 1988). In addition to its critical endocrine actions on the pituitary, GnRH has important effects in the brain, where it modulates certain aspects of reproductive behavior (Moss and Dudley, 1989). The essential role of GnRH in mammalian reproductive physiology has led to extensive investigations of the structure/function of this peptide hormone and the synthesis of more than 2000 analogs, some of which have found widespread clinical applications (see review by Karten and Rivier (1986)). These studies have exhaustively documented the relative importance of each of the 10 GnRH residues in various aspects of receptor binding and bioactivity. Similar investigations have determined which residues are required for metabolic stability and have shed light on the in vivo enzymatic degradation of the hormone. The majority of the reported residue substitution and peptide modification experiments have not extensively addressed questions concerning the intrinsic chemical reactivity of functional groups on GnRH and its analogs. However, knowledge of intrinsic residue reactivity can provide significant information relating to hormonal mode of action and can be valuable in interpreting results obtained from three-dimensional structural analysis. The use of the avidin-biotin system to study molecular and biological interactions has expanded significantly over the last decade (see reviews by Bayer and Wilchek (1980) and Wilchek and Bayer (1990)). Biotinylation reagents have been widely employed to acylate amino groups on lysyl and a-amino-terminal residues of proteins and peptides (Hofmann and Finn, 1985; Hochhaus et al., 1988; Wilchek and Bayer, 1988). These published reports have emphasized that the specificity of NHS-biotin esters is directed to amino groups (Yen et al., 1989), and there have been no previous reports of O-acetylation by these reagents. In this report, we describe that, in the case of GnRH and related analogs, considerable O-acetylation can occur under reaction conditions normally employed for peptide biotinylation. In addition to describing the

\textsuperscript{1}The abbreviations used are: GnRH, gonadotropin-releasing hormone; NHS-biotin, N-hydroxysuccinimide esters of biotin; sulfosuccinimidyl-6-(biotinamido) hexanoate; HPLC, high performance liquid chromatography; HEPES, N-2-hydroxyethylpiperazine N-2-ethanesulfonic acid; LH, luteinizing hormone; FSH, follicle-stimulating hormone; EMIP, epidermal mitosis inhibitory peptide; MS, mass spectrometry; MS/MS, tandem mass spectrometry; FAB, fast atom bombardment; CID, collision-induced dissociation; MS-1 and MS-2, the first and second spectrometers of a tandem high resolution mass spectrometer; [M + H]\textsuperscript{+}, protonated molecular ion; [m+Na]\textsuperscript{+}, sodium adduction; RIA, radioimmunoassay.
modification, purification, and detailed structural analysis of these unexpected derivatives, we have also evaluated the relative bioactivity of the various monobiotinylated GnRH species isolated by HPLC. Furthermore, we have compared the results of acylation of GnRH and its analogs with unrelated but structurally similar peptides. Finally, we discuss the implications of these findings in the general design and interpretation of peptide biotinylation investigations that require the preparation of biotinylated peptide ligands.

**EXPERIMENTAL PROCEDURES**

**Materials**—Synthetic [d-Lys^6]GnRH was purchased from Bachem, Inc. (Torrance, CA) or from Peninsula Laboratories, Inc. GnRH, des-Gly^10-[d-Trp^7]GnRH, and EMIP were obtained from Peninsula. Eledoisin and physalaemin were purchased from Bachem. HCl (6 N; Sequest grade) and sulfosuccinimidyl-6-(biotinamido) hexanoate (sulfo-NHS-Ahx-biotin) were purchased from Pierce Chemical Co. Sulfo-NHS-Ahx-biotin corresponds to Pierce’s NHS-LC-biotin. Ultrapure guanidine HCl was purchased from Schwarz/Mann Biotech. Trifluoroacetic acid (reagent grade), acetonitrile (HPLC grade), hydroxylamine hydrochloride, sodium bicarbonate, and boric acid (all certified grade) were obtained from Fisher. Vydac C18 reversed-phase (218 TP) semipreparative columns (5-µm particle size; 300-A pore diameter; 10 mm × 25 cm) and direct connect guard columns with Vydac semiconductor (5-µm particle size) were produced by Applied Science Labs. Animals were obtained from Harlan Sprague-Dawley, Inc. Bovine serum albumin (RIA grade, fraction V) and all tissue culture media, antibiotics, and other culture reagents were obtained from Sigma. Radioimmunoassay kits containing standard hormone preparations (NIDDK-yl-HRP-2 and NIDDK-FSH-RP-2) and antisera were provided by the National Institute of Diabetes and Digestive and Kidney Diseases.

**Biotinylation**—Biotinylation reactions were conducted in 50 mM sodium bicarbonate (250 µl) at peptide concentrations of 1 mg/ml, pH 8.2, containing sulfo-NHS-Ahx-biotin at indicated reagent: peptide molar ratios. The sulfo-NHS-Ahx-biotin was added in a small volume of 0.1% trifluoroacetic acid to minimize excessive reagent hydrolysis by water. The reaction mixtures were gently shaken every 5–10 min during the course of the biotinylation. In time course biotinylation experiments, aliquots of the reaction mixture were removed at various intervals as indicated, acidified to pH 3–4 with 0.1% trifluoroacetic acid, and rapidly frozen. All aliquots and reaction mixtures were stored at −20 °C until HPLC analysis. For biotinylation reactions in the presence of guanidine HCl, identical aliquots of GnRH were dissolved in 0.1 M sodium bicarbonate containing 6 M guanidine HCl, pH 8.2, and incubated at 25 or 50 °C for 5 min. These solutions were then biotinylated for 40 min at 25 °C with sulfo-NHS-Ahx-biotin. The entire reaction mixtures were then acidified and subjected to HPLC analysis. A separate set of experiments was conducted to examine the stability of the derivatives. Biotinylated peptide fractions were heated at 37 °C for 2 h in 0.1% trifluoroacetic acid and re-analyzed by C18 reversed-phase HPLC. As a point of information, we have reanalyzed some of the biotinylated peptide derivatives by amino acid analysis. Preincubation medium was removed from 48-h dispersed cells and replaced with 1 ml of incubation media that contained the peptide derivative to be tested (three wells/peptide). After incubation for 4 h at 37 °C, media were removed, and centrifuged at 10,000 × g at 4 °C, and supernatant fluids were collected and stored at −20 °C prior to radioimmunoassay.

**RESULTS**

The primary structures of the peptides used in these studies are shown in Table I.

**Biotinylation of [d-Lys^6]GnRH**—Reaction of [d-Lys^6]GnRH with 190-µl of 0.1 M succinimidyl 6-(biotinamido) hexanoate (sulfo-NHS-Ahx-biotin) was carried out in 50 mM sodium bicarbonate (250 µl) at peptide concentrations of 1 mg/ml, pH 8.2, containing sulfo-NHS-Ahx-biotin at indicated reagent: peptide molar ratios. The sulfo-NHS-Ahx-biotin was added dry to a buffered (pH 8.2) solution of 0.1% trifluoroacetic acid and added to a buffered (pH 8.2) solution of 0.1% trifluoroacetic acid. After incubation for 4 h at 37 °C, media were removed, and centrifuged at 10,000 × g at 4 °C, and supernatant fluids were collected and stored at −20 °C prior to radioimmunoassay.

**Hydroxyamine Reaction**—Hydroxyamine hydrochloride solutions (0.8 M in 0.1 M boric acid) were adjusted to pH 9.2 with NaOH. Aliquots of HPLC-purified, biotinylated peptide were dissolved in a small volume of 0.1% trifluoroacetic acid and added to the buffered hydroxyamine solution. The reaction was allowed to proceed for 4 h at 25 °C. Control incubations were carried out in 0.1 M boric acid, pH 9.2. The reaction mixtures were then acidified with trifluoroacetic acid and analyzed by C18 reversed-phase HPLC. HPLC—Lyophilized peptides and reaction mixtures were dissolved in 0.1% trifluoroacetic acid and applied to a Vydac C18 reversed-phase HPLC semipreparative column. Peptides were eluted at 25 °C with a linear gradient of solvent A (0.1% trifluoroacetic acid) and solvent B (0.1% trifluoroacetic acid containing 80% acetonitrile containing 0.1% trifluoroacetic acid) at a flow rate of 1.75 ml/min. Our standard linear gradient for the GnRH analogs and their biotinylated derivatives was 0–45% solvent B over 120 min. An identical gradient was used during chromatography of EMIP. For HPLC of eledoisin and physalaemins, a linear gradient of 12–57% solvent B over 120 min was employed. The column eluate was monitored at 215 nm, and 1-min fractions were collected. Fractions were pooled based on absorbance and were subjected to amino acid compositional analysis, mass spectrometry, and bioassay.

All fractions and aliquots were lyophilized and stored at −20 °C.

**Amino Acid Analysis**—Samples were hydrolyzed with 6 N HCl in vacuo at 107 °C for 22–24 h. Amino acid compositional analysis was carried out on a Beckman 121MB analyzer employing single column methodology on Beckman W-2 resin or on a Beckman 6300 analyzer. Alternatively, samples were analyzed directly with a Bioanalytical Systems 420H derivatizer-hydrorizer that provided on-line hydrolysis and phenylthiocarbamyl derivatization. The Applied Biosystems 420H analyzer was employed for the quantification of the 6-amino hexanoic acid spacer arm, which provided information about the extent of biotinylation of the various peptide derivatives (Smith et al., 1991).

**Mass Spectrometry**—Mass spectrometric analyses were performed at the Mass Spectrometry Facility, Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts. FAB/MS analyses of the GnRH-related biotinylated derivatives were carried out in the first of two mass spectrometers of a tandem high resolution mass spectrometer (JEOL HX110/HX110) as previously described (Barber et al., 1981). Tandem MS was carried out using all four sectors of the JEOL HX110/HX110, essentially according to Sato et al. (1987). Briefly, the CID of protonated peptide molecules, selected with MS-1, took place in the field-free region after Br, thus operating both MS-1 and MS-2 as double-focusing instruments. The CID mass spectra were recorded with 100 Hz filtering at a rate that corresponds to a scan from m/z 0 to 6000 in 1.5 min. MS-1 was operated at a resolution set to transmit only the 14C species of the protonated peptide molecule to be analyzed. MS-2 was operated at a resolution of 3000 and was calibrated with a mixture of Cs, Na, KI, Rb, and LiCl.

**Bioassays**—Pituitary glands of intact, adult male Sprague-Dawley rats were removed within 1 min of sacrifice and placed in ice-cold Ca2+- and Mg2+-free Hanks’ balanced salt solution supplemented with 14% bovine serum albumin, 25 mM HEPES, and gentamicin sulfate (100 µg/ml). The neurointermediate lobes were removed, and the anterior pituitaries were washed, quartered, diced with a sterile razor blade, and washed again with Hanks’ salt solution. The tissues were gently agitated for 25 min at 25 °C in a solution of 0.3% trypsin containing 2.5 µg DNAase/anterior pituitary, and then mechanically disrupted with a Pasteur pipette (40X) in 1 ml of Medium-199 containing trypsin inhibitor and DNAase (each 25 µg/ml). Dispersed cells were recovered by low speed centrifugation, resuspended, and examined for cell number and viability using a hemacytometer and the trypan blue exclusion test (Freshney, 1983). Viabilities greater than 90% and cell counts of 1 × 107 cells were routinely observed. Cells were cultured in a moisturized atmosphere of air:CO2 (95:5) at 37 °C in Medium-199 containing Earle’s balanced salts, 2 mM CaCl2, 13 mM HEPES, 17 mM NaHCO3, 1000 units/ml penicillin, 100 µg/liter streptomycin, and 10% FCS in a humidified incubator at 5% CO2.

HPLC-purified GnRH, GnRH analogs, and their biotinylated derivatives were dissolved in a small volume of 0.1% trifluoroacetic acid and diluted to their indicated final concentrations in culture medium in which the fetal calf serum was replaced by bovine serum albumin (100 µg/ml). All biotin-labeled mass spectrometers were determined by amino acid analysis. Preincubation medium was removed from 48-h dispersed cells and replaced with 1 ml of incubation media that contained the peptide derivative to be tested (three wells/peptide). After incubation for 4 h at 37 °C, media were removed, and centrifuged at 10,000 × g at 4 °C, and supernatant fluids were collected and stored at −20 °C prior to radioimmunoassay.

Measurements of LH and FSH in the cell media were performed using specific radioimmunoassays as previously described (Parkening et al., 1982; Westlund et al., 1984), employing kits and standard hormones supplied by the National Institutes of Health (see “Materials”). The sensitivities of these assays were approximately 0.3 ng for LH and 15 ng for FSH. Intra-assay variation was less than 10% for LH and less than 7% for FSH. Neither the synthetic peptides nor their biotinylated derivatives, at the concentrations used in these studies, interfered with the RIA. Statistically significant (p < 0.05) inhibition of LH or FSH response by derivatives were determined by two-way analysis of variance for repeated measurements, followed by a Newman-Keul’s test (Bruning and Kintz, 1977).
O-Acylation of Serine in GnRH and Related Peptides

Table I

Primary structures of relevant peptides biotinylated in this study.

| Peptide                  | Formula                  |
|-------------------------|--------------------------|
| GnRH                    | <Glu-His-Tyr-Glu-Leu-Arg-Pro-Gly> |
| [D-Lys’]GnRH            | <Glu-His-Tyr-Glu-Leu-Arg-Pro-Gly> |
| Des-Gly< [D-Trp’]GnRH   | <Glu-His-Tyr-D-Trp-Leu-Arg-Pro> |
| Eledoisin               | <Glu-Pro-Ser-Lys-Ala-Phe-Ile-Gly-Leu-Met> |
| Physalaemin             | <Glu-Ala-Asp-Pro-Asn-Lys-Phe-Tyr-Gly-Leu-Met> |
| EMIP                    | <Glu-Glu-Ser-Gly>         |

Sequence taken from Matsuo et al. (1971).
Amide-containing carboxyl terminus.
Ethylamide-containing carboxyl terminus.
Sequence taken from Bernardi et al. (1966).
Sequence taken from Reichelt et al. (1987).

GnRH with sulfo-NHS-Ahx-biotin at a 4:1 molar ratio of reagent:peptide for 10 min at 25 °C resulted in six major fractions, as evidenced by HPLC (Fig. 1A). Fraction A1 appeared at the same position as unmodified [D-Lys’]GnRH, whereas fractions A2-A6 appeared to be chemically distinguishable biotinylated derivatives. The multiple peptide derivatives illustrated in Fig. 1A were consistently observed during the chromatography of many different biotinylation reaction mixtures carried out under various reaction conditions. Amino acid compositional analysis indicated that each fraction contained a peptide identical in composition with the original [D-Lys’]GnRH. When [D-Lys’]GnRH was reacted with sulfo-NHS-Ahx-biotin at a 20:1 molar ratio of reagent:peptide for 60 min at 25 °C, no unbiotinylated peptide (fraction A1) remained, and the biotinylated derivatives represented by fractions A2, A3, and A5 were absent (Fig. 1B).

Fig. 1C shows the chromatography of pooled aliquots of peptide fraction A6 that was obtained by HPLC after treatment of the modified peptide with 0.1% trifluoroacetic acid for 2 h at 37 °C. The acid treatment had no effect on the HPLC elution time of fraction A6. Identical acid treatment of fractions A2-A5 also had no effect on their HPLC elution times (results not shown).

To establish whether or not the biotin moieties were attached via ester linkages to hydroxyamino acids, the HPLC-purified material in the major fractions was reacted with hydroxylamine at alkaline pH and subsequently rechromatographed. These experiments demonstrated that the biotinylated derivatives in all the fractions except for A1 and A3 (Fig. 1) were hydroxylamine-labile. A representative example of the experiments with hydroxylamine is illustrated in Fig. 2. Fig. 2A shows the repeat HPLC elution of pooled material from fraction A6 (Fig. 1A) incubated in buffer without hydroxylamine. Reaction with hydroxylamine (Fig. 2B) resulted in a shift in the elution time of the fraction A6 peptide to a position corresponding to fraction A3. HPLC analyses of the derivatives in the other fractions both before and after hydroxylamine treatment were obtained in a similar fashion. The results of these analyses are summarized in Table II.

Mass Spectrometric Analysis—HPLC-purified peptide products from the biotinylations of [D-Lys’]GnRH, fractions A1-A6 (Fig. 1), were analyzed by both FAB/MS and CID tandem MS. The mass of the molecular ions observed in the spectra established the number of biotin moieties present in each peptide derivative. Fig. 3 illustrates the FAB/MS of [D-Lys’]GnRH (fraction A1; Fig. 1) and its major biotinylated derivatives (fractions A2-A6). The spectrum of derivative A1 (Fig. 3A) indicated a protonated molecule at m/z 1255.8, consistent with the structure of [D-Lys’]GnRH shown in Table I. The spectra of derivatives A2 and A3 (Fig. 3, B and C) indicated major ions at m/z 1592.9 and were in agreement with the calculated value for a monobiotinylated [D-Lys’] GnRH peptide, whereas the spectrum of fraction A4 (Fig. 3D) was consistent with a dibiotinylated peptide (m/z 1933.0). Fraction A5 (Fig. 3E) also contained a dibiotinylated peptide (m/z 1933.0), the majority of which was present as the sodium adduct (m/z 1955.0). Fraction A6 (Fig. 3F) contained a tribiotinylated species of [D-Lys’]GnRH, as indicated by the presence of three biotin moieties.

FIG. 1. Reversed-phase HPLC purification of [D-Lys’] GnRH and reaction products after reaction with sulfo-NHS-Ahx-biotin. A, 4:1 reagent:peptide molar ratio for 10 min at 25 °C. The pooled fractions were designated A1-A6, as indicated. B, 20:1 reagent:peptide molar ratio for 60 min at 25 °C. C, rechromatography of pooled aliquots of fraction A6 after treatment with 0.1% trifluoroacetic acid for 2 h at 37 °C.

FIG. 2. Reversed-phase HPLC analysis of pooled aliquots of fraction A6 (Fig. 1A) after reaction with buffered hydroxylamine, pH 9.2. The acetonitrile gradient was identical with that shown in Fig. 1A. A, elution profile of purified fraction A6 in control buffer. The elution positions of [D-Lys’]GnRH and its major biotinylated derivatives are indicated. B, elution profile after reaction of purified fraction A6 with buffered hydroxylamine. The deacylated peptide eluted at a position corresponding to that of peak A3.
whereas the latter is that of a peptide containing biotinylated lysine. These still contained the biotin substituent, indicating bio-lysine.

The fragment nomenclature used here has been described previously (Biemann, 1988). The biotinylated derivative from HPLC fraction A2 (FAB spectrum shown in Fig. 3B) yielded a CID spectrum that was similar to that shown in Fig. 2272.1. Representative CID/MS/MS analyses for the identification of the modified amino acid residues in specific biotinylated species are shown in Fig. 4. The CID spectrum shown in Fig. 4A, obtained from the protonated peptide molecule at m/z 1253.8 (Fig 3A) is that of unmodified [D-Lys6]GnRH from HPLC fraction A1. As expected, this spectrum exhibited the characteristics of a peptide containing one or more basic amino acids at or near the carboxyl terminus. Due to the presence of arginine as the third amino acid from the carboxyl terminus, the spectrum was dominated by the w, and x ions. The fragment nomenclature used here has been described previously (Biemann, 1988).

The biotinylated derivative from HPLC fraction A2 (FAB spectrum shown in Fig. 3B) yielded a CID spectrum that was only compatible with reaction at serine (Fig. 4B). The region below m/z 400 was similar to that shown in Fig. 4A. While the w, ion was the same because it had lost the side chain of lysine, the occurrence of other lysine-containing carboxy-terminal ions, z, y, and x, demonstrated that this residue was not modified. This conclusion was also supported by the abundant w, ion. However, the most compelling reason for assigning this isomer the structure of a peptide biotinylated at serine was the abundant ion at m/z 1235.5, which indicated the conversion of serine to dehydroalanine as a result of the elimination of the biotin moiety and the loss of the serine hydroxyl. The CID spectrum illustrated in Fig. 4C is that of the isomeric protonated peptide at m/z 1592.3 shown in Fig. 3C (from HPLC fraction A3). The relatively wide region devoid of significant peaks indicated that the long chain biotin moiety was attached somewhere in the center of the peptide, consistent with modification at the D-Lys6 residue. Since the formation of w, ions involved elimination of part of the lysine side chain, w, had lost the biotin moiety and, therefore, was of the same mass as the derivatized peptide. The m/z values of the peaks designated y, z, x, and w, required that all of these still contained the biotin substituent, indicating biotinylation at D-Lys6. The two CID spectra shown in Fig. 4, B and C, therefore, demonstrate that the former represents that of a peptide in which acylation of the serine has taken place, whereas the latter is that of a peptide containing biotinylated lysine.

### Table II

| HPLC retention summary of biotinylated GnRH-related derivatives after hydroxylamine treatment |
|---------------------------------------------------------------|
| Before hydroxylamine | After hydroxylamine |
|----------------------|---------------------|
| [D-Lys6]GnRH         | A1                  |
|                      | A2                  |
|                      | A3                  |
|                      | A4                  |
|                      | A5                  |
|                      | A6                  |
| Des-Gly10,[D-Trp]GnRH| B1                  |
|                      | B2                  |
|                      | B3                  |
|                      | B4                  |
|                      | C1                  |
|                      | C2                  |
|                      | C3                  |
|                      | C4                  |

* Refers to the position of the numbered peaks in Figs. 1 and 5. Hydroxylamine unreactive derivatives have identical elution times.

### Biotinylation of Peptides Unrelated to GnRH—To compare the reactivity of the hydroxyamino acids in GnRH and its analogs with the reactivity of similar residues in unrelated peptides, we performed a series of biotinylation reactions with the peptides eledoisin and physalaemin. These peptides, in addition to being structurally similar to GnRH, allowed us to compare the relative occurrence of O-acylation and N-acylation within each peptide. Eledoisin and physalaemin are both 11 residues in length, have pyroglutamyl residues as amino termini, and have amidated C-terminal residues. Both peptides contain a single lysine, with eledoisin having a single serine at position 3 and no tyrosine, whereas physalaemin has one tyrosine at position 8 and no serine. The reaction of synthetic eledoisin with sulfo-NHS-ε-Ahx-biotin at a 4:1 reagent:peptide molar ratio for 10 min at 25 °C resulted in one major biotinylated species, as illustrated in the HPLC profile in Fig. 6A. Glycine compositional analysis confirmed that fraction D1 was unmodified eledoisin, whereas fraction D2 was a monobiotinylated derivative. Reaction of fraction D2 with hydroxylamine had no effect on HPLC retention time, indicating that the biotin moiety was attached to the Lys residue at position 4. There was no indication of significant O-acylation of the seryl residue.

Fig. 6B shows the HPLC elution of synthetic physalaemin after reaction with sulfo-NHS-ε-Ahx-biotin at a 4:1 reagent:peptide molar ratio for 10 min at 25 °C. Amino acid compositional analysis indicated that fraction E1 was unmodified physalaemin, whereas fractions E2 and E3 were monobiotinylated derivatives. Fraction E4 was a dibiotinylated species. Reaction of fraction E2 with hydroxylamine had no effect on its HPLC elution time, indicating that it was monobiotinylated on Lys6. By similar reasoning, we deduced that fraction E3 was monobiotinylated on Tyr8 and fraction E4 was dibiotinylated on Lys6 and Tyr8.

The time course biotinylation of specific residues in [D-Lys6]GnRH, eledoisin, and physalaemin are shown in Fig. 7. The results illustrated in Fig. 7 were generated by reacting...
equivalent amounts of peptide with sulfo-NHS-Ahx-biotin under identical reaction conditions. In each peptide reaction, approximately 60% of the lysyl residues had reacted by 10 min. Strikingly, the reactivity of Ser\(^4\) in [D-Lys\(^6\)]GnRH was significantly high and somewhat paralleled that of the lysyl residue. Over 45% of the serine was biotinylated within 10 min (75% relative to lysine). The reactivity of the [D-Lys\(^3\)]GnRH serine was in marked contrast to the results obtained from biotinylation of eleidoisin, in which case less than 2% of the seryl residue was acylated in the first 10 min of the reaction. By comparison, the reactivity of the tyrosyl residues in [D-Lys\(^3\)]GnRH and physalaemin was relatively low and roughly equivalent.

A molar comparison of serine acylation after reaction of [D-Lys\(^6\)]GnRH, eleidoisin, and EMIP with sulfo-NHS-Ahx-biotin at a relatively high 20:1 reagent:peptide molar ratio is shown in Fig. 8. Under these conditions, all of Ser\(^4\) in the GnRH analog was biotinylated, whereas only about 6% of Ser\(^4\) in eleidoisin was modified. Virtually no seryl acylation was detected in the pentapeptide EMIP, which contained 1 seryl residue and no available amino groups.

**Biotinylation under Denaturing Conditions**—Biotinylation of GnRH with sulfo-NHS-Ahx-biotin at 25 °C in the presence of 6 M guanidine HCl, after heating the peptide in the denaturing solution at 100 °C for 5 min, reduced O-biotinylation substantially (Fig. 9D) when compared with the control (Fig. 9A). Incubation of GnRH in 6 M guanidine HCl for 1 h at 25 or 50 °C significantly reduced O-biotinylation, but appreciable amounts of monobiotinyl serine and monobiotinyl tyrosine were still evident (Fig. 9, B and C) when compared with the biotinylation reaction with prior heating at 100 °C (Fig. 9D). Incubation of [biotinyl-Ser\(^4\)]GnRH in biotinylation reaction buffer containing 6 M guanidine HCl for 40 min prior to HPLC analysis showed no evidence of deacylation (results not shown).

**Bioactivity Studies**—Fig. 10 illustrates the effects of native GnRH, [D-Lys\(^6\)]GnRH, and their monobiotinylated derivatives on the release of LH from dispersed rat anterior pituitary cells. GnRH caused a significant release of LH at concentrations greater than 1 nM, whereas [biotinyl-Ser\(^4\)]GnRH and [biotinyl-Tyr\(^6\)]GnRH (Fig. 5B, derivatives C2 and C3, respectively) were effective only at 100 nM, the highest concentration tested (Fig. 10A). However, even at this concentration, the monobiotinylated derivatives were only 40% as active as unmodified GnRH. By comparison, a notable difference in bioactivity of the monobiotinylated derivatives of [D-Lys\(^3\)]GnRH was observed (Fig. 10B). Both [D-Lys\(^6\)]GnRH and [biotinyl-Lys\(^3\)]-[D-Lys\(^6\)]GnRH significantly increased LH release at and above concentrations of 1 nM. However, the bioactivity of [biotinyl-Ser\(^4\)]-[D-Lys\(^6\)]GnRH was significantly reduced and, as with [biotinyl-Ser\(^4\)]GnRH, only achieved significant LH release at a concentration of 100 nM. It should be noted that the bioactivity of [biotinyl-Lys\(^3\)]-[D-Lys\(^6\)]GnRH was consistently greater than that of the unmodified analog
in repeated bioassays. In general, the release of FSH from the pituitary cultures after stimulation with both peptides and their biotinylated derivatives closely paralleled the results from the LH radioimmunoassays (results not shown).

**DISCUSSION**

Reaction of [D-Lys"GnRH with a sulfonated N-hydroxysuccinimide ester of biotin generated multiple forms of biotinylated peptide (Fig. 1). Since [D-Lys"GnRH contained only one available amino group, it was evident that other functional groups on the peptide had reacted with the sulfo-NHS-Ahx-biotin reagent. Because we observed that relatively large amounts of these unexpected derivatives appeared throughout the course of the reaction, we undertook an extensive structural characterization of all major reaction products. This characterization involved HPLC purification, amino acid compositional analysis, hydroxylamine reaction, and analysis by both FAB/MS and CID tandem MS. The results of these analyses clearly demonstrated that, in addition to the expected modification at D-Lys" biotin moieties were also attached to [D-Lys"GnRH via ester linkages on Ser4 and Tyr'. Evidence of similar O-acylation was obtained from separate experiments with a second GnRH analog, des-Gly["-Trp"GnRH (Fig. 5A), as well as with synthetic GnRH (Fig. 5B), neither of which contain reactive amino groups. In addition, O-acylated derivatives of GnRH and analogs were obtained after reaction with the nonsulfonated NHS-Ahx-biotin reagent (results not shown).

The most striking finding from the analyses of the biotinylation reaction products was the evidence of a highly reactive seryl residue (Ser4) in GnRH and both analogs. In the case of [D-Lys"GnRH, a significant amount of [biotinyl-Ser4]-[D-Lys"GnRH was generated in the first 10 min of reactions carried out at a 4:1 reagent:peptide molar ratio (Fig. 1). In this GnRH analog, the serine reactivity closely paralleled the reactivity of the lysyl residue (Fig. 7). In addition, after 1 h at a reagent:peptide molar ratio of 20:1, all of the serine in [D-Lys"GnRH could be modified (Figs. 1B and 8). By comparison, the reactivity of Tyr' to biotinylation was considerably less than that of Ser4 in both [D-Lys"GnRH and des-Gly["-Trp"GnRH (Figs. 1 and 5A), although tyrosine modification increased at higher molar ratios of reagent:peptide. For example, at a reagent:peptide molar ratio of 10:1, the yield of [biotinyl-Tyr']GnRH approached that of [biotinyl-Ser4]GnRH (Fig. 5B). We found no evidence of monobiotinylated [D-Lys"GnRH modified on Tyr'. This derivative may have been one of the minor HPLC components not evaluated. It is important to note that none of the major O-acylated peptide derivatives were short lived reaction intermediates. In fact, these biotinylated peptides were stable
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**O-Acylation of Serine in GnRH and Related Peptides**

![CID mass spectra](image)

**FIG. 4.** Representative CID mass spectra of [D-Lys⁶]GnRH and biotinylated derivatives. **A**, unmodified [D-Lys⁶]GnRH, [M + H]⁺ m/z = 1253.8; **B**, biotinylated at Ser⁴, [M + H]⁺ m/z = 1592.9; **C**, biotinylated at D-Lys⁶, [M + H]⁺ m/z = 1592.9. Fragment nomenclature is from Biemann (1988).

**FIG. 5.** Reversed-phase HPLC purification of des-Gly⁶-[D-Trp⁹]GnRH, native GnRH, and reaction products after reaction with sulfo-NHS-coumarin-biotin. **A**, des-Gly⁶-[D-Trp⁹]GnRH; **B**, synthetic GnRH. Peptides were reacted at a 10:1 reagent:peptide molar ratio for 20 min at 25 °C. Fractions B1 and C1 represent unmodified peptides. Fractions B2 and C2 were biotinylated at Ser⁴, B3 and C3 at Tyr⁷, and B4 and C4 at both Ser⁴ and Tyr⁷.

During dry storage at −20 °C for several months, as evidenced by rechromatography by HPLC. Moreover, as a further test of stability, biotinylated [D-Lys⁶]GnRH derivatives were treated with 0.1% trifluoroacetic acid for 2 h at 37 °C and subsequently rechromatographed. As typified in Fig. 1C for the tribiotinylated species (fraction A6), no significant desaclylation of any of the [D-Lys⁶]GnRH derivatives was observed. This acid stability contrasts with the relative instability of acylated tyrosyl residues, as previously reported in the case of pepsinogen succinylation (Gounaris and Perlmann, 1967).

Although the occurrence of O-acylation has been reported under reaction conditions typically employed for N-acylation for a number of reagents, this is the first report of such reactivity for the N-hydroxysuccinimide esters of biotin. In fact, many recent in-depth reports on the structural characterization of biotinylated derivatives of peptide hormones, such as that of β-endorphin (Hochhaus et al., 1988), parathyroid hormone (Newman et al., 1989), interleukin-1β (Yem et al., 1989), porcine relaxin (Bullesbach and Schwabe, 1990), and egg-laying hormone (Knock et al., 1991), indicated no evidence of O-acylation even when a large molar excess of reagent was employed (e.g. 10:1 reagent:protein for interleukin-1β). In the case of the 36-residue egg-laying hormone of *Aplysia*, which has 1 seryl, 2 threonyl, and 1 tyrosyl residue, O-acylation using NHS-coumarin-biotin was specifically investigated (Knock et al., 1991). Moreover, some reports categorically state that the NHS-biotin reagents are highly specific for amino groups (Yem et al., 1989). The fact that the biotinylated residues in many of these modified peptides have been identified and no evidence of O-biotinylation was reported emphasizes the likelihood that the serine residues in GnRH...
TABLE III

Stoichiometry of biotinylation of GnRH-related derivatives

| HPLC fraction | Mass* (m/z) | Biotin† (mol/mol) | Residues biotinylated‡ |
|---------------|------------|-------------------|------------------------|
| [D-Lys6]GnRH  |            |                   |                        |
| A1           | 1253.8     | 0                 |                        |
| A2           | 1592.9     | 1 Ser4            |                        |
| A3           | 1592.9     | 2 d-Lys6          |                        |
| A6           | 1933.0     | 3 Ser4, d-Lys6    |                        |
| A7           | 1933.0     | 2 Tyr6, d-Lys6    |                        |
| A8           | 2272.1     | 3 Ser4, Tyr6, d-Lys6 |                   |
| Des-Gly[45]-[D-Trp5]GnRH |            |                   |                        |
| B1           | 1282.6     | 0                 |                        |
| B2           | 1621.9     | 1 Ser4            |                        |
| B3           | 1621.8     | 1 Tyr6            |                        |
| B4           | 1961.2     | 2 Ser4, Tyr6      |                        |
| GnRH         |            |                   |                        |
| C1           | 1182.6     | 0                 |                        |
| C2           | 1521.8     | 1 Ser4            |                        |
| C3           | 1521.8     | 1 Tyr6            |                        |
| C4           | 1641.9     | 2 Ser4, Tyr6      |                        |

* From FAB/MS analysis.
† Established by FAB/MS analysis and amino acid compositional analysis (Smith et al., 1991).
‡ Established by FAB/MS, and/or CID/MS/MS, and hydroxylamine reaction.
§ Unmodified synthetic peptide.
¶ Each biotin moiety adds 339.1 to the mass.

and its analogs are uniquely reactive toward activated esters of biotin. Further evidence in this regard was obtained when we compared the time course of biotinylation of serine and lysine in eledoisin and [D-Lys6]GnRH (Fig. 7). Under identical reaction conditions that demonstrated comparable lysyl reactivity in both peptides, over 45% of Ser4 in [D-Lys6]GnRH was acylated in the first 10 min, whereas less than 2% of the eledoisin serine was biotinylated. Moreover, when equivalent reaction mixtures of [D-Lys6]GnRH, eledoisin, and EMIP were biotinylated at a high reagent:peptide molar ratio of 20:1, all of Ser4 in [D-Lys6]GnRH was acylated, whereas only ~6% of the eledoisin serine and <1% of the EMIP serine was modified (Fig. 8). The result from the biotinylation of EMIP was especially notable, since this pentapeptide contained the active site sequence (Asp-Ser-Gly) typically found in serine proteases (Blow et al., 1969).

The unusually high reactivity of the Ser4 residue in GnRH and its analogs prompted us to investigate O-acylation under denaturing conditions to inquire whether or not the observed reactivity was associated with conformation. Carrying out the biotinylation reaction in the presence of 6 M guanidine HCl after heating the peptide at 100 °C adversely affected O-biotinylation (Fig. 9D), indicating that the acylation of serine and tyrosine was dependent on peptide conformation. It was noteworthy that denaturation of GnRH in 6 M guanidine HCl at lower temperatures (25 and 50 °C) was incomplete after 1 h (Fig. 9B and C), suggesting that the native conformation of GnRH surprisingly was relatively stable under these conditions. We found no indication that the guanidine treatment itself hydrolyzed ester linkages between biotin and GnRH, and the observed decrease in biotinylation with increasing temperature gave added evidence that the reduction in O-biotinylation was not due to decacylation.

A likely explanation for the observed superreactivity of the seryl residue is increased nucleophilicity of the hydroxyl oxy-
cultured cells were collected and assayed for LH by RIA. The results are shown as the average LH release ± S.E. of triplicate determinations. The fraction designations correspond to those in Fig. 5B. A, reaction of GnRH with sulfo-NHS-αAhx-biotin for 40 min at 25°C without guanidine; B, same as A, but after 60 min in 6 M guanidine HCl at 25°C; C, same as A, but after 60 min in 6 M guanidine HCl at 50°C; D, same as A, but heated in 6 M guanidine HCl for 5 min at 100°C.

The unusually high reactivity of Ser4 in GnRH, coupled with the significant loss of bioactivity associated with Ser4 biotinylation, or with residue substitution, invites further inquiry concerning the potential role of this residue in the mechanism of action of GnRH. For example, is the observed reactivity of Ser4 primarily the result of its participation in hydroxylation.

All of the monobiotinylated O-acetylated derivatives of [D-Lys5]GnRH and GnRH were assayed for their ability to release LH from cultured pituitary cells. In these assays, O-acetylation of both analogs shifted the dose-response curve to the right by approximately 2 orders of magnitude (Fig. 10A). Since the Ser4 residue of GnRH is one of 5 highly conserved residues in the GnRH family of peptides (Sherwood, 1987), modifications at this position appear to be critical to bioactivity. An [Ala4]GnRH analog was reported to have only 5% of the activity of native GnRH (Geiger et al., 1972; Yanoihara et al., 1973). Similarly, modification of the Tyr4 residue also results in marked decreases in bioactivity (Yanoihara et al., 1973). These reported potency estimates for Ser4 and Tyr4 replacement analogs are consistent with our bioassay results. In addition, similar results of reduced potency were obtained from in vivo studies using O-acetylated derivatives of GnRH (Baba et al., 1971).

Biotinylation of the Lys5 residue in [D-Lys5]GnRH had no detrimental effect on the ability of the peptide to stimulate LH secretion, and in fact, increased LH secretion when compared with the unmodified peptide (Fig. 10B). A similar result was reported by Tibolt and Childs (1985), using a different biotinylating reagent that did not contain an extended spacer arm.

The unusually high reactivity of Ser4 in GnRH, coupled with the significant loss of bioactivity associated with Ser4 biotinylation, or with residue substitution, invites further inquiry concerning the potential role of this residue in the mechanism of action of GnRH. For example, is the observed reactivity of Ser4 primarily the result of its participation in

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**Fig. 9. Reversed-phase HPLC purification of the biotinylated derivatives of GnRH after reaction with sulfo-NHS-αAhx-biotin following treatment with 6 M guanidine HCl.**

**Fig. 10. Bioassay of GnRH, [D-Lys5]GnRH, and their monobiotinylated derivatives.** Dispersed anterior pituitary cells from adult male rats were exposed to the HPLC-purified peptides at the indicated concentrations for 4 h at 37°C. Supernatant fluids from the cultured cells were collected and assayed for LH by RIA. The results shown are the average LH release ± S.E. for three culture wells. A, LH release after exposure to unmodified GnRH (●), [biotinyl-Ser4]GnRH (●), [biotinyl-Tyr4]GnRH (●); B, LH release after exposure to unmodified [D-Lys5]GnRH (○), [biotinyl-Ser4]-[D-Lys5]GnRH (●), or [biotinyl-d-Lys5]GnRH (●).
maintenance of conformation or could this residue have a more direct role in GnRH action? Clearly, the results obtained herein concerning the intrinsic reactivity of Ser4 in GnRH raise important new questions concerning the potential role of this residue in structure/function considerations of GnRH. Knowledge of the unique reactivity of Ser4 will be of value in interpreting the three-dimensional structure of GnRH when it becomes available. For example, the determination of the unique reactivity of Ser4 of the chymotrypsinogen family of serine proteinases to dipropyl fluorophosphate was highly important in developing the hypothesis that the observed charge relay in chymotrypsin that centered at Ser4 was part of the catalytic mechanism of the active site (Balls and Jansen, 1952; Schaffer et al., 1953; Blow et al., 1969). Although synthetic residue substitution experiments and similarly site-directed mutagenesis give us valuable information about structure and/or function, they do not address intrinsic reactivity of residue side chains.

In summary, chemical modification of GnRH and related peptides using sulfo-NHS-Ahx-biotin yielded a number of biotinylated peptides with various combinations of modifications on lysyl, seryl, and tyrosyl residues. A time course of biotinylation was notable in demonstrating that the seryl residue at position 4 was especially reactive. Under identical reaction conditions, seryl residues in selected, structurally similar peptides unrelated to GnRH demonstrated little, if any, reactivity. Peptide denaturation experiments gave strong evidence that the unique reactivity of Ser4 is in all likelihood related to conformational parameters. Biotinylation of [D-Lys4]GnRH at α-Lys4 resulted in a derivative with increased in vitro activity, whereas acylation of the single seryl residue in GnRH and [D-Lys4]GnRH, and the single tyrosyl residue in GnRH, greatly reduced bioactivity. The procedures we have described for isolating and chemically characterizing peptide biotinylation reaction products, including O-acylated derivatives, have general applicability to other peptides and can be carried out on relatively small amounts of material. In addition, the occurrence of O-acylation during biotinylation using NHS-biotin reagents could explain the unexpected loss of biologics activity in those cases where N-acylated derivatives were expected to remain bioactive. These results emphasize the potential value of employing NHS-biotin esters as general chemical modification reagents for seryl and tyrosyl hydroxyl groups. There is a reasonable likelihood, as evidenced by results described herein, that these reagents have a fortuitous reactivity that is capable of discriminating between especially reactive side chain hydroxyl groups.

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