BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **218,** 377–382 (1996)
Article No. 0066

Acylation of Peptide Hydroxyl Groups with the Bolton–Hunter Reagent

Brian T. Miller

Department of Anatomy & Neurosciences, The University of Texas Medical Branch, Galveston, Texas 77555-1043

Received November 20, 1995

Reaction of the decapeptide gonadotropin releasing hormone (GnRH) with the Bolton–Hunter reagent produced a single major derivative. Mass spectrometric analysis of this derivative at M-Scan Corporation revealed that O-acylation of the Ser^4 hydroxyl had occurred. Formation of the O-acylated Ser^4 derivative was dependent on the presence of the His^2 residue in GnRH. Similar experiments with several unrelated peptides revealed that the Bolton–Hunter reagent will readily acylate hydroxyl groups on serine, tyrosine, and threonine side chains located two positions from a histidine residue (e.g., His-X-Ser). Such O-acylated peptides can be formed under mild reaction conditions and appear to be relatively stable. Recognition of this sequence-specific O-acylation can be critical when labeling peptides with the Bolton–Hunter reagent and when interpreting experiments in which such modified peptides are used.

The Bolton-Hunter reagent (N-succinimidyl 3-(4-hydroxyphenyl) propionate) has been used for several years to chemically modify proteins and peptides (1–5). The utility of this reagent resides in its ability to label peptides and proteins with radioiodinated hydroxyphenyl moieties. The modifying group is incorporated via amide bond formation with lysine ϵ-amino functions and N-terminal α-amino groups (1). Thus, most proteins and peptides with few or no tyrosine side chains, the traditional sites of direct iodination, can be derivatized to generate radioactive probes. Use of the Bolton-Hunter reagent can also be necessary when modification of native tyrosine residues will result in diminished receptor binding or bioactivity. Moreover, acylation with the Bolton-Hunter reagent can at times yield radiolabeled antibodies with higher immunoreactivity than those prepared by using chloramine-T (1,4–5).

The Bolton-Hunter reagent is an NHS-ester, one of a class of compounds commonly used either to modify primary amines or as cross-linking agents (6). A reported advantage of using these esters is their specificity for reacting with primary amino groups (6,7). However, NHS-esters routinely used to biotinylate peptide amines will readily react with hydroxyl groups on serine residues located in linear His-X-Ser or Ser-X-His sequences (8). Such unexpected reactions can have dramatic effects on the bioactivity of derivatized peptides, and, if unrecognized, could result in significant error in the interpretation of experimental results (9). The present study was undertaken to determine whether Ser, Tyr, or Thr residues located in His+2 or His−2 positions would react with the Bolton-Hunter reagent to yield stable O-acylated derivatives.

MATERIALS AND METHODS

Synthetic peptides and chemical reagents. Synthetic [Ala^2]GnRH was obtained from Chiron Mimotopes Peptide Systems (San Diego, CA). All other synthetic peptides were purchased from Sigma (St. Louis, MO), Peninsula Laboratories (Belmont, CA), or Bachem (Torrance, CA). Bolton-Hunter reagent and hydroxylamine hydrochloride were purchased from Sigma. All solvents were HPLC grade or sequanal grade.

Acylation reactions. For individual reactions, 5 nmol peptide were dissolved in 0.2 mL of 0.05 M sodium phosphate buffer (pH 8 except where indicated). Bolton-Hunter reagent (5–500 nmol dissolved in 0.025 mL DMF or DMSO) was added and the reaction continued at room temperature until terminated by the addition of 1 mL 0.1% TFA. Reaction mixture products were resolved by HPLC using a linear gradient of increasing acetonitrile in 0.1%TFA. All HPLC separations were

Abbreviations used: BH, Bolton-Hunter, DMF, dimethylformamide; DMSO, dimethyl sulfoxide; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; NHS, N-hydroxysuccinimide; TFA, trifluoroacetic acid; X, any amino acid.

0006-291X/96 $12.00

achieved with Vydac analytical C18 reversed-phase columns on a Beckman Analytical Gradient workstation as reported (10). Column eluate was monitored at 215 nm, and 1-mL fractions were collected. Deacylation of peptide derivatives was carried out using 1 M hydroxylamine hydrochloride in 0.1 M borate buffer, pH 9.2, for 4–5 hours at room temperature as described (10). Amino acid analyses of the various peptide species were performed on an Applied Biosystems, Inc. automatic analyzer as reported (9).

Mass spectrometry. Identification of the specific amino acid side chains in GnRH acylated by the Bolton-Hunter reagent was carried out at M-Scan Corporation, West Chester, PA. Sequence ions were produced by FAB-MS on a ZAB-2SE high field mass spectrometer (VG Analytical, Manchester, UK) and assigned as previously described (10).

RESULTS AND DISCUSSION

When GnRH was reacted for one hour with the Bolton-Hunter reagent (8:1 reagent:peptide molar ratio) and the reaction mixture subjected to HPLC, a single peptide derivative was observed (Fig. 1, peak 3). As mammalian GnRH contains no lysine residues and has a blocked N-terminus (pyroglutamic acid), the observed modification could not have been the result of amide bond formation. This was confirmed when the derivatized peptide was deacylated by treatment with hydroxylamine (not illustrated). Identification of the precise site of modification on the peptide was accomplished by employing FAB-MS (10–11). Fig. 2 illustrates the spectrum generated from the HPLC-purified GnRH derivative. The principal pseudomolecular $(M + H)^+$ ion at m/z 1330 was consistent with presence of GnRH derivatized by a single Bolton-Hunter moiety, whereas the signal at m/z 1184 represented the intact peptide ion that had lost its modifying group during FAB. The observed fragmentation pattern was dominated by C-terminal sequence ions, as might be expected due to the peptide's amide C-terminus and blocked N-terminus. Assignment of the indicated X-, Y-, and Z-type C-terminal sequence ions, depicted at the top of the figure, strongly support the conclusion that the hydroxyl group on Ser^4 was the sole site of modification. No evidence of His^2 or Tyr^5 acylation was observed.

The ability of the Ser^4 hydroxyl in GnRH to react with the Bolton-Hunter reagent was dependent upon the presence of the His^2 residue. When His^2 in GnRH was replaced by Ala, no reaction product was observed despite the use of a 100:1 reagent:peptide molar ratio (Fig. 3B). Under identical reaction conditions, the Ser^4 in native GnRH was quickly derivatized (Fig. 3A). It is important to note that biotinylation of the Ser^4 hydroxyl in GnRH and its analogs with NHS-biotin esters also requires the presence of a His^2 residue (8).

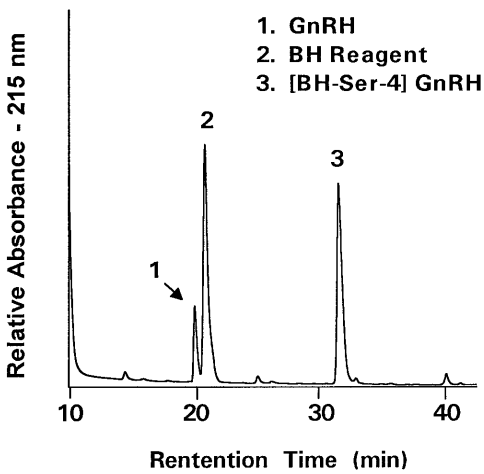

FIG. 1. HPLC of the reaction mixture of GnRH and the Bolton–Hunter (BH) reagent (8:1 reagent:peptide molar ratio, pH 8, 1 hour). Under these conditions, over 70% of the peptide was converted into a single derivative (peak #3). Identification of Ser^4 as the site of modification was accomplished by FAB-MS (See Fig. 2). HPLC gradient: 15–60% acetonitrile containing 0.1% TFA developed over 2 hours.

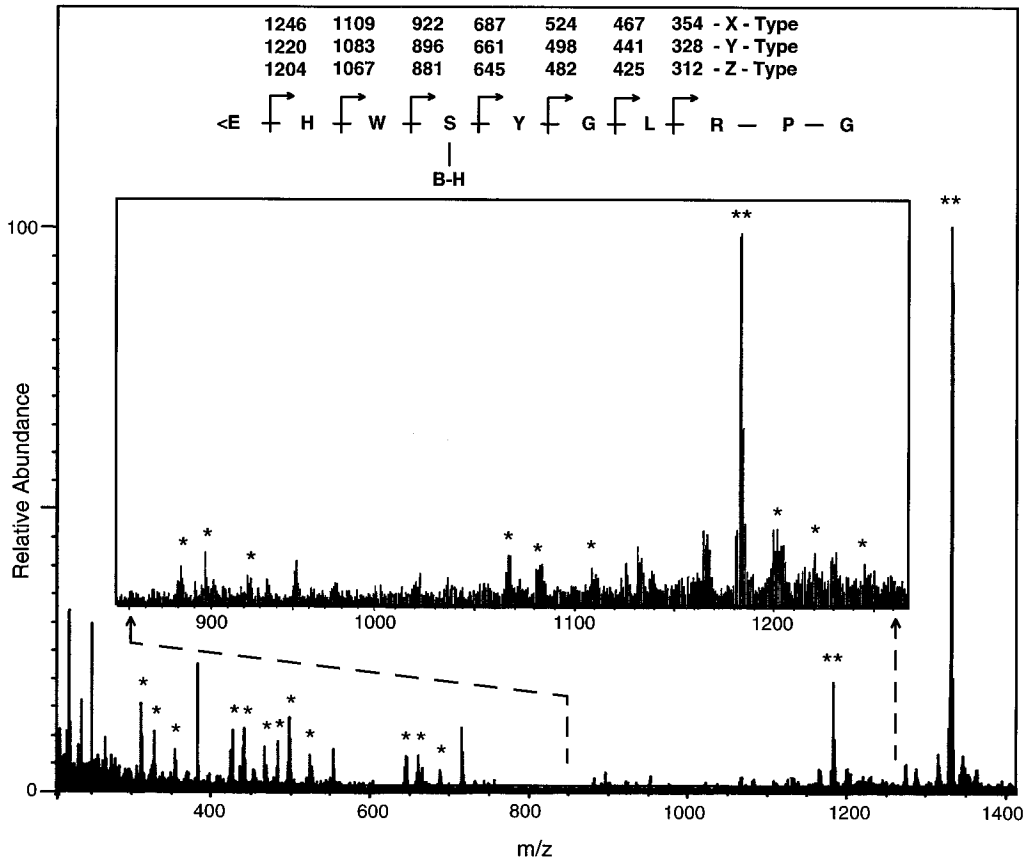

FIG. 2. FAB-MS of the major derivative from reaction of the Bolton–Hunter reagent with GnRH (peak #3 in Fig. 1). The boxed inset shows an expanded scale between m/z 850 and m/z 1310. The $(M+H)^+$ pseudomolecular ions at m/z 1330 and m/z 1184 indicated by the double asterisks represent, respectively, the modified GnRH peptide and the intact GnRH peptide that has lost the Bolton–Hunter moiety. The peptide sequence at the top of the figure illustrates the assignment of the observed fragmentation ions labeled by single asterisks. The spectrum is consistent with a GnRH peptide modified only at Ser^4. (B–H = Bolton–Hunter moiety).

Reducing the pH of Bolton-Hunter reactions favors the acylation of α-amino groups over ϵ-amino groups (3). To assess the effect of pH on the relative acylation of ϵ-amino groups versus hydroxyl functions, we used the Bolton-Hunter reagent to modify [$D\text{-}Lys^6$]GnRH, an analog of GnRH that contains one primary amino group on its $D\text{-}Lys^6$ residue. Structural characterization of the modified peptide reaction products was accomplished by amino acid analysis and hydroxylamine treatment of the purified derivatives. After a 30 min reaction at pH 8 (10:1 reagent:peptide molar ratio), two major derivatives were observed, one monoacylated and one diacylated (Fig. 4B). Under these conditions, all the $D\text{-}Lys^6$ had been modified by the reagent and approximately 45% of the modified peptide products also contained acylated Ser^4 residues. An equivalent reaction at pH 7 proceeded more slowly, but yielded nearly equal amounts of modified Ser^4 and $D\text{-}Lys^6$ side chains (Fig. 4A). Therefore, the decrease in pH affected the ϵ-amino group reactivity more than the serine hydroxyl reactivity. Consequently, the pH 7 reaction products contained significant amounts of peptide monoacylated at Ser^4 (Fig. 4A, peak 3). These results indicated that changes in reaction pH can alter the relative proportions of modified serine hydroxyl and lysine ϵ-amino groups in Bolton-Hunter reaction mixtures. Reducing the pH of the reaction mixture below 6 essentially abolished O-acylation of the Ser^4 hydroxyl in [$D\text{-}Lys^6$]GnRH, a finding consistent with the involvement of a deprotonated histidine imidazole (8).

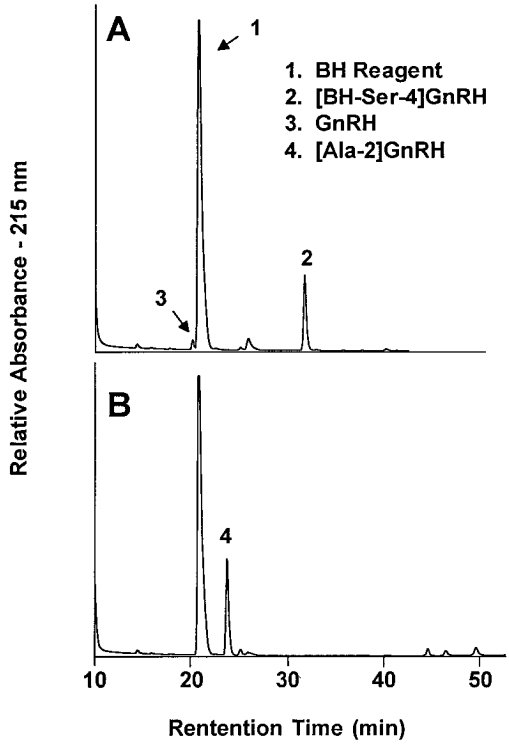

FIG. 3. HPLC of reaction mixtures of the Bolton–Hunter reagent and **(A)** GnRH or **(B)** [Ala^2]GnRH (both 100:1 reagent:peptide molar ratio, pH 8, 30 min). Although virtually all the native peptide has been derivatized on Ser^4 under these conditions, no acylation of the [Ala^2] analog was observed. HPLC gradient as in Fig. 1.

In addition to GnRH and its analogs, several unrelated peptides were reacted with the Bolton-Hunter reagent to determine whether the Ser, Tyr, or Thr hydroxyls in these peptides could be acylated under relatively mild conditions (1:1 to 10:1 reagent:peptide molar ratios for 1–2 hours at pH 8). Table 1 lists the primary structures of the peptides used in this study. Evidence of significant O-acylation was obtained by HPLC of the reaction mixtures before and after treatment with hydroxylamine. This analysis permitted determination of which peptides contained reactive hydroxyls, and which contained hydroxyl groups that were unmodified after exposure to the reagent (Table 1). The results of these experiments strongly suggested that Ser, Tyr, or Thr side chains located two positions from a His residue can undergo significant O-acylation by the Bolton-Hunter reagent under relatively mild conditions. Peptides without such His-containing sequence triads were not acylated on their hydroxyl functions. For example, reaction of angiotensin II produced a major hydroxylamine-sensitive derivative, in addition to the expected N-terminal modification. By contrast, under identical reaction conditions, [p-aminophenylalanine^6]angiotensin II, an analog lacking His, was modified only on the α-amino group. Experiments with the peptide hormone galanin demonstrated that positioning the His-X-Ser triad in the middle of a medium-sized peptide did not noticeably impede O-acylation.

The O-acylated derivatives formed in these reactions did not appear to be short-lived, labile species. Rechromatography of some of the modified peptides after several hours at neutral or acidic pH showed little evidence of loss of the O-linked Bolton-Hunter moieties. However, we have not yet determined the relative stability of the ester linkages on these derivatives.

Together with the demonstration of sequence-specific peptide O-acylation by NHS-esters of biotin (8–10), the present results emphasize that peptides containing hydroxy amino acids in a His

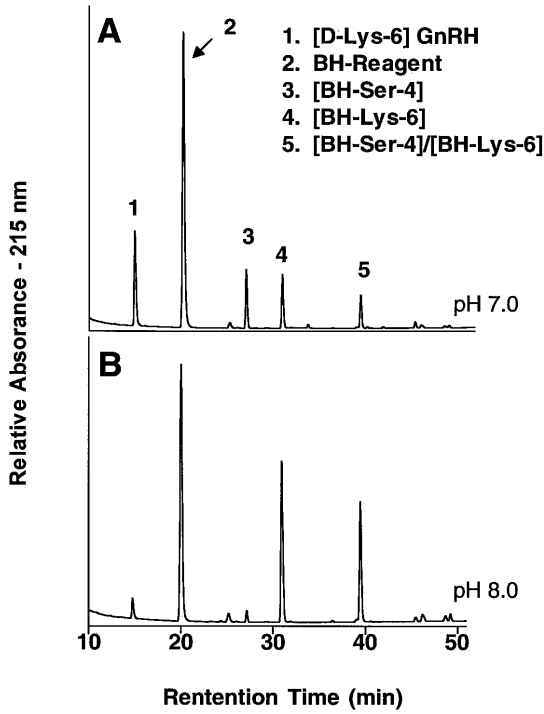

FIG. 4. HPLC of reaction mixtures of the Bolton–Hunter reagent and [D-Lys^6]GnRH at **(A)** pH 7 or **(B)** pH 8 (both 10:1 reagent: peptide molar ratio, 30 min). Whereas essentially all the D-Lys^6 residue was modified at pH 8, relatively more O-acylation occurred at pH 7. The slower reaction of pH 7 also produced more monoacylated derivatives. HPLC gradient as in Fig. 1.

+ 2 or His − 2 position will, in all likelihood, react with NHS-esters and other electrophilic reagents to form O-acylated products. This elevated hydroxyl reactivity could be the result of hydrogen bond formation between the hydrogen of the hydroxyl group and a specifically positioned imidazole nitrogen. Alternatively, O-acylated hydroxyls could be generated by acylimidazole transfer from a

TABLE 1
Primary Structures of Peptides Reacted with the Bolton–Hunter Reagent

Peptides with reactive hydroxyl groups*	
GnRH	⟨Glu-*His-Trp-Ser*-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
[D-Lys^6]GnRH	⟨Glu-*His-Trp-Ser*-Tyr-D-Lys-Leu-Arg-Pro-Gly-NH_2
Galanin	Gly-Trp-Thr-Leu-Asn-Ser-Ala-Gly-Tyr-Leu-Leu-Gly-Pro-His-Ala-Val-Gly-Asn-*His-Arg-Ser*-Phe-Ser-Asp-Lys-Asn-Gly-Leu-Thr-Ser
Angiotensin II	Asp-Arg-Val-*Tyr-Ile-His*-Pro-Phe
VHLTP**	Val-*His-Leu-Thr*-Pro
Peptides with unreactive hydroxyl groups	
[Ala^2]GnRH	⟨Glu-Ala-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
Eledoisin	⟨Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2
EMIP***	⟨Glu-Glu-Asp-Ser-Gly
[p-aminophenylalanine^6] Angiotensin II	Asp-Arg-Val-Tyr-Ile-(p-amino-Phe)-Pro-Phe

* The putative sites of O-acylation are on the hydroxy-bearing residues located within the underlined linear amino acid triads. Human GnRH, galanin, and angiotensin peptide sequences were used in these experiments.

** Amino-terminal pentapeptide of the beta chain of hemoglobin.

*** Epidermal mitosis inhibitory pentapeptide.

derivatized histidine side chain. However, we have, to date, not observed the formation of acyl-imidazole intermediates, or any His derivative, when modifying peptides with the Bolton-Hunter reagent or with NHS-biotin esters.

Dozens of bioactive peptides and hundreds of peptide analogs contain Ser, Tyr, or Thr residues in a His + 2 or His − 2 position. Obviously, failure to appreciate the probability of significant O-acylation in these peptides could lead to errors when purifying modified peptides by HPLC and when interpreting results from experiments with the peptide derivatives. It is likely, for example, that reaction of the Bolton-Hunter reagent with avian pancreatic polypeptide (APP) will result in both N-acylation at the amino terminus and O-acylation of the C-terminal tyrosine which is located in a /His-Arg-Tyr/ sequence (12). Such C-terminal modification might explain the loss of APP binding affinity observed after reaction of the hormone with this reagent (13). Nevertheless, the predictability of sequence-specific O-acylation and apparent stability of the resultant ester linkages raise the possibility that distinct O-acylated derivatives could be synthesized and used to augment studies of the structure and function of selected bioactive peptides.

ACKNOWLEDGMENTS

I thank Dr. Mark Rogers of M-Scan Corporation for his expert analysis and interpretation of the FAB-MS of acylated GnRH. I also acknowledge and thank Dr. Alex Kurosky, Director of the UTMB Protein Chemistry Laboratory where the amino acid analyses of the various peptides and peptide derivatives were performed. My thanks also go to Ms. Rosalinda Rodriguez for her technical assistance and to Ms. Kay Smith and Ms. Regina Minton for their contributions in the preparation of the illustrations. I am grateful to Dr. T. J. Collins for helpful discussions.

REFERENCES

1. Bolton, A. E., and Hunter, W. M. (1973) *Biochemical Journal* **133,** 529–539.
2. Kimmel, J. R., and Pollack, H. G. (1981) *Endocrinology* **109,** 1693–1699.
3. Gaudriault, G., and Vincent, J. P. (1992) *Peptides* **13,** 1187–1192.
4. Kermode, J. C., and Thompson, B. D. (1980) *J. Endocrinology* **84,** 439–447.
5. Sturgeon, C. M., Beynon, L., Seth, J., and Chisholm, G. D. (1983) *Ann. Clin. Biochem.* **20,** 112–115.
6. Lundblad, R. L. (1995) Techniques in Protein Modification, p. 154, CRC Press, Boca Raton, FL.
7. Yem, A. W., Zurcher-Neely, H. A., Richard, K. A., Staite, N. D., Heinrikson, R. L., and Deibel, M. R. (1989) *J. Biol. Chem.* **264,** 17691–17697.
8. Miller, B. T., and Kurosky, A. (1993) *Biochem. Biophys. Res. Commun.* **196,** 461–467.
9. Miller, B. T., Collins, T. J., Nagle, G. T., and Kurosky, A. (1992) *J. Biol. Chem.* **267,** 5060–5069.
10. Miller, B. T., Rogers, M. E., Smith, J. S., and Kurosky, A. (1994) *Analyt. Biochem.* **219,** 240–248.
11. Roepstorff, P., and Fohlman, J. (1984) *Biomed. Mass. Spectrom.* **11,** 601–603.
12. Kimmel, J. R., Hayden, L. J., and Pollock, H. G. (1975) *J. Biol. Chem.* **250,** 9369–9376.
13. Adamo, M. L., and Hazlewood, R. L. (1992) *Neuropeptides* **21,** 225–230.