Vol. 196, No. 1, 1993 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1993 Pages 461-467

ELEVATED INTRINSIC REACTIVITY OF SERYL HYDROXYL GROUPS WITHIN THE LINEAR PEPTIDE TRIADS HIS-Xaa-SER OR SER-Xaa-HIS

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Received September 7, 1993

Summary: Chemical modification studies of peptide hormones and random peptides have revealed that seryl hydroxyl groups had enhanced reactivity toward acylating reagents when they occurred in the linear triads His-Xaa-Ser or Ser-Xaa-His (Xaa = any amino acid). O-acylation of serine within these triads was achieved by reaction with N-hydroxysuccinimide esters of biotin (NHS-biotin) and succinic anhydride. Seryl residues not occurring in His-Xaa-Ser/Ser-Xaa-His triads showed no reactivity toward NHS-biotin under reaction conditions described. Results of histidine replacement studies and studies of the pH dependence of O-biotinylation indicated that the increased nucleophilicity of the seryl hydroxyl group was due to intramolecular interaction between the seryl and histidyl residues. Our findings provide strong evidence that such triads represent novel consensus motifs in peptides.

We previously demonstrated that several peptides related to GnRH contain a Ser^4 hydroxyl group that has unusually elevated intrinsic reactivity as evidenced by reaction with NHS-ϵAhx-biotin (1). The Ser^4 residue in these peptides is readily O-acylated by NHS-ϵAhx-biotin, even though this reagent was previously reported to specifically acylate only primary amino groups (2). In subsequent investigations it was striking that GnRH antagonists that contained amino acids other than histidine in position 2 could not be O-acylated under conditions described. In view of these results, we have undertaken studies described herein, in which we have investigated the general phenomenon of hydroxyl group reactivity of seryl residues in peptides as a function of intramolecular histidine proximity. Interactions of histidine and serine, which are well known to play critical roles in many enzymic active site mechanisms, may also be important in the bioactivity of some peptides.

Abbreviations: GnRH, gonadotropin releasing hormone; EMIP, epidermal mitosis inhibiting pentapeptide; Xaa, any amino acid; NHS-ϵAhx-biotin, N-biotinyl 6-aminohexanoyl N-hydroxysuccinimide ester; TFA, trifluoroacetic acid; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment-mass spectrometry.

0006-291X/93 $4.00

MATERIALS AND METHODS

Synthetic Peptides and Chemical Reagents - GnRH, eledoisin, and related peptides were purchased from Peninsula Labs or Bachem (Torrance, CA); amylin[14-24], frog neurotensin, and EMIP were obtained from Peninsula. Katacalcin was purchased from Sigma Chemical Co. Synthetic model peptides were obtained from Boston Bioscience, Inc.

Biotinylation - Peptides were biotinylated at pH 8.0 and 25° C with NHS-ϵAhx-biotin (Sigma) essentially as previously described (3). Reactions were stopped by the addition of 1 M glycine-HCl, pH 3.0, and the mixtures were immediately injected onto a Vydac C18 reversed-phase HPLC column. Collected fractions of interest were pooled and subjected to amino acid analysis and peptide sequence analysis as reported (4,5). Kinetic studies of the biotinylation of seryl hydroxyls were carried out as described previously (3).

Succinylation - Amylin[14-24] was dissolved in 0.1 M phosphate buffer, pH 8.0. Succinic anhydride (Sigma) was dissolved in acetonitrile and a 40-fold molar excess was added to the dissolved peptide to initiate the reaction. Additional reagent (40-fold molar excess) was added to the reaction mixture every 5 min, while the pH was maintained near 8.0 with NaOH. The reaction was terminated after 30 min by addition of TFA and the entire mixture was applied to an HPLC column (3). Succinylated peptide fractions were analyzed by FAB-MS.

HPLC - All chromatographic separations were carried out on Vydac analytical C18 reversed-phase columns as previously reported (3). The solvents used for gradient fractionation were 0.1% TFA and 0.1% TFA in acetonitrile.

Mass Spectrometry - The specific amino acid residues modified in biotinylated and succinylated peptides were identified by FAB-MS on a ZAB-2SE high field mass spectrometer (VG Analytical, Manchester, UK) at M-Scan Corporation, West Chester, PA.

RESULTS AND DISCUSSION

As a consequence of our studies with GnRH and its peptide analogs (1), we have examined the chemical reactivity of seryl hydroxyl groups in a number of unrelated bioactive peptides (Table 1). Several of these contained more than one seryl residue. Reaction of these peptides with NHS-ϵAhx-biotin was followed by HPLC separation of the reaction products. Biotinylated derivatives were characterized by amino acid analysis, by FAB-MS, and by reaction with hydroxylamine (1). The results of these experiments demonstrated that, in addition to N-biotinylation of primary amino groups, certain seryl residues were also O-biotinylated by NHS-ϵAhx-biotin. In every case, we found that when seryl O-acylation occurred, the seryl residue was located in the third position within a His-Xaa-Ser triad (Table 1). Seryl residues located in other positions in the same peptide were unreactive even when they were adjacent to histidine as in the case of amylin[14-24] and neurotensin (Table 1). In addition, other control peptides containing serine but not histidine were also unreactive to NHS-ϵAhx-biotin (Table 1).

To further characterize seryl reactivity within peptides, we conducted biotinylation studies on four synthetic model peptides (Table 1). Sequences were selected for random coil conformation. Representative HPLC fractionations of two biotinylation reaction

Table 1. Primary structures of peptides containing His-Xaa-Ser linear triads (underlined) O-acylated by reaction with NHS-ϵAhx-biotin

Biologically Relevant Peptides	
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·
Des-Gly^{10}-[D-Trp^6]GnRH	<Glu-His-Trp-Ser*-Tyr-D-Trp-Leu-Arg-Pro-NH-CH_2-CH_3·
[Ala^2]GnRH	<Glu-Ala-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2·
Amylin[14-24]	·Asn-Phe-Leu-Val-His-Ser*-Ser-Asn-Asn-Phe-Gly·
Neurotensin (Frog)	<Glu-Ser-His-Ile-Ser*-Lys-Ala-Arg-Arg-Pro-Tyr-Ile-Leu·
Katacalcin	·Asp-Met-Ser-Ser-Asp-Leu-Glu-Arg-Asp-His-Arg-Pro-His-Val-Ser*-Met-Pro-Gln-Asn-Ala-Asn·
Eledoisin	<Glu-Pro-Ser-Lys-Asp-Ala-Phe-Ile-Gly-Leu-Met-NH_2·
EMIP	<Glu-Glu-Asp-Ser-Gly·
Synthetic Model Peptides	
	<Glu-Asn-His-Ala-Ser*-Phe-Val-NH_2·
	<Glu-Asn-Ala-Ala-Ser-Phe-Val-NH_2·
	<Glu-Asn-Ser*-Ala-His-Phe-Val-NH_2·
	<Glu-Asn-Ser-Ala-Ala-Phe-Val-NH_2·

*Indicates residues O-acylated by NHS-ϵAhx-biotin; unmarked seryl residues were not reactive.

mixtures (reactive and unreactive) are shown in Fig.1. All reaction components, including starting products and reaction products, were readily separated by HPLC. As shown in Fig. 1, the model peptide lacking the histidyl residue was unreactive to NHS-ϵAhx-biotin whereas the model peptide with the His-Ala-Ser triad was readily O-acylated. Plots of the pseudo-first-order reaction kinetics for the biotinylation of these two peptides are shown in Fig. 2. Combining both peptides, <ENHASFV and <ENAASFV, in the same reaction mixture yielded identical results, indicating that O-acylation was not influenced by

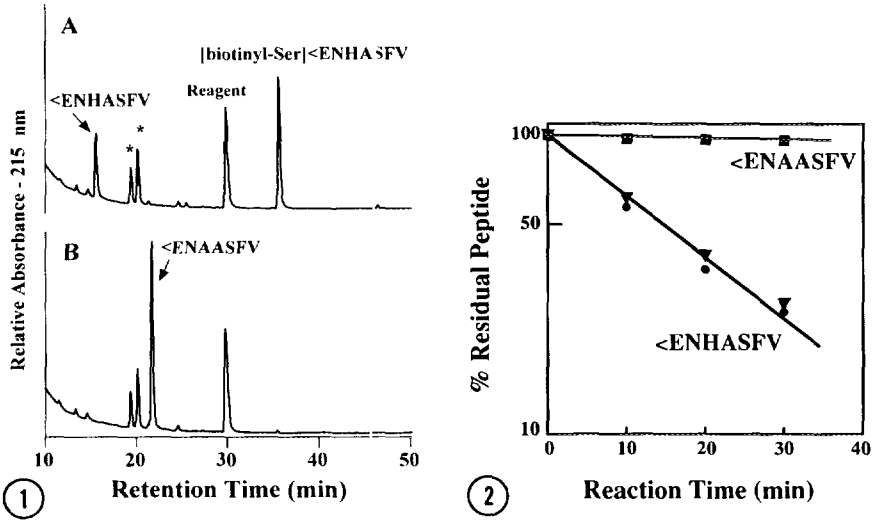

Fig. 1. HPLC chromatograms of biotinylation reaction mixtures of (A) <ENHASFV and (B) <ENAASFV. The chromatograms shown are from 30-min time-points of the biotinylation reaction time-course at 25° C. Reaction mixtures contained 10 nmol of peptide and a 25:1 molar excess of reagent:peptide in 50 mM sodium phosphate buffer, pH 8. The biotinylated-Ser[5] derivative from <ENHASFV is shown in panel A; no corresponding biotinylated-Ser[5] derivative was observed after biotinylation of <ENAASFV (panel B). The peak labeled "Reagent" is intact NHS-ϵAhx-biotin; the peaks labeled with asterisks are hydrolyzed products of NHS-ϵAhx-biotin.

Fig. 2. Pseudo-first-order reaction kinetic plots of the biotinylation of <ENHASFV and <ENAASFV. 10 nmol of peptide was reacted with NHS-ϵAhx-biotin at a ratio of 25:1 reagent:peptide at 25° C. Duplicate determinations were made for each time-point.

intermolecular interactions. Interestingly, comparable results were obtained after biotinylation of two additional model peptides, <ENSAHFV and <ENSAAFV, in which the His-Xaa-Ser triad sequence was reversed to Ser-Xaa-His. The seryl residue in <ENSAHFV was readily O-acylated by NHS-ϵAhx-biotin, whereas the seryl hydroxyl in <ENSAAFV was unreactive (data not shown). Significantly, biotinylation of the seryl residue in <ENHASFV was pH dependent as shown in Fig. 3 and gave a sigmoidal response with a pK of 6.65 that was consistent with the ionization of the imidazole of histidine.

Preliminary kinetic studies established that the pseudo-first-order rate constant (k_1') for O-biotinylation of the seryl residue in <ENHASFV was about 8.25 X 10^{-4} s^{-1}. We had previously determined that an average k_1' for N-acylation of peptidyl lysyl ϵ-amino groups was about 1.15 X 10^{-2} s^{-1} under comparable reaction conditions (3). Therefore, the N-acylation rate was about 14X faster than the observed O-acylation rate stated above.

To inquire whether or not the observed enhanced seryl reactivity was reagent specific for NHS-ϵAhx-biotin, we employed several different biotinylating reagents, including

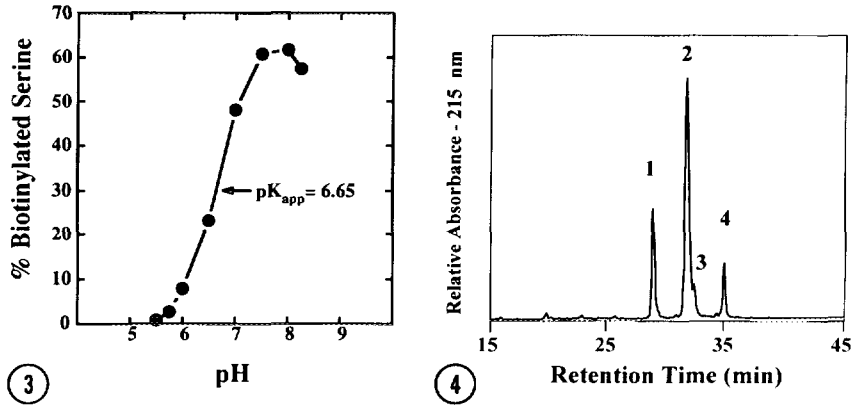

Fig. 3. Effect of pH on biotinylation of Ser[5] in <ENHASFV. The total percent of seryl residues biotinylated were determined after reaction for 20 min at 25°C in sodium phosphate buffer at the indicated pH values. The apparent pK is 6.65.

Fig. 4. HPLC profile of the reaction products resulting from succinylation of an amylin[14-24] fragment (see Table 1). Reaction time was 20 min at 25°C. Succinic anhydride (40:1 reagent:peptide) was added every 5 min.

sulfonated and non-sulfonated esters both with and without spacer arms. All of these reagents were capable of O-acylating seryl hydroxyls in His-Xaa-Ser triads (data not shown). In addition, we reacted some peptides with succinic anhydride, a reagent known to acylate hydroxyamino acid residues (6). Fig. 4 illustrates the HPLC separation of the reaction products from a 30-min succinylation of the amylin[14-24] fragment (Table 1). The major reaction product occurred in peak 2 and was identified by FAB-MS to be a disuccinylated peptide in which succinyl moieties were attached to the α-amino group on Asn[1] and the hydroxyl group on Ser[7]. The HPLC retention times of peaks 2, 3, and 4 after reaction with hydroxylamine shifted to that of peak 1, the N-acylated derivative (results not shown). Peak 4 was the trisuccinylated derivative and peak 3 was likely the disuccinylated peptide acylated at Asn[1] and Ser[6]. The relative degree of succinylation under the reaction conditions described was 100% for Asn[1], 71% for Ser[7], and 16% for Ser[6]. These results clearly indicated that Ser[7] was more reactive than Ser[6]; however, further kinetic analyses will be required to document this more fully. Succinic anhydride indicated to be a more aggressive O-acylating agent when compared to NHS-ϵAhx-biotin which typically does not O-acylate seryl residues unless they are especially reactive.

The observed increase in serine reactivity within the His-Xaa-Ser triad did not appear to be due to general base catalysis by histidine. Free imidazole (up to 100-fold molar excess) did not catalyze seryl biotinylation in peptides that lacked His and had no significant effect on the O-biotinylation kinetics of peptides containing a reactive His-Xaa-Ser triad

(data not shown). Bodanszky *et al.* (7) had reported a side reaction during peptide synthesis involving histidine-catalyzed O-acylation of serine after reaction with N-hydroxysuccinimide esters of *tert*-butyloxycarbonyl-ß-benzyl-L-aspartic acid. This O-acylation could also be catalyzed by the addition of free imidazole. However, their anhydrous reaction conditions were considerably different from those of our reactions.

Our current hypothesis is that the observed seryl hydroxyl reactivity within the His-Xaa-Ser triad is due to a specific base catalysis mechanism (8). In this hypothesis, hydrogen bonding can form between an imidazole nitrogen and the hydrogen of the hydroxyl group on the seryl residue. This would increase the nucleophilicity of the hydroxyl oxygen and give rise to elevated intrinsic reactivity. Specific base catalysis could therefore be due to the action of the histidyl imidazole which is uniquely poised to interact with the serine by the conformational features of the triad. Preliminary modeling of a number of His-Xaa-Ser and Ser-Xaa-His sequences using energy minimization programs has indicated that His/Ser interactions can occur in such triads. Specific base catalysis is consistent with the fact that εAhx-biotin is a poor leaving group (9).

An alternative hypothesis for the observed enhanced reactivity within the triad motif involves nucleophilic catalysis by imidazole (9). In this mechanism the imidazole functions as a nucleophile and first acts on the reagent to form an N-acylimidazole intermediate. This N-acylimidazole intermediate would then O-acylate the seryl hydroxyl group (10). The observed reactivity could be enhanced by proximity effects (8) and would be sequence specific. Further studies will be required to delineate the reaction mechanism definitively.

At present, the biochemical function of the His-Xaa-Ser triad motif is an open question; however, it is reasonable to extrapolate from existing evidence that these reactive triads in bioactive peptides may be playing significant roles in biologic phenomena. For example, a properly positioned nucleophilic oxygen would be an ideal candidate to either stabilize peptide-receptor binding or initiate receptor activation. Activated seryl hydroxyl functions could react with specific electrophilic targets on receptor molecules.

In conclusion, there is significant evidence from both bioactive and synthetic random peptides to indicate that linear triads of His-Xaa-Ser or Ser-Xaa-His represent novel elements of peptide structure in which the seryl hydroxyl has enhanced nucleophilicity. We have not yet evaluated the reciprocal effect of the seryl residue on histidine reactivity which may also be influenced. Finally, and most obvious, these linear triad motifs are in part reminiscent of the Asp-His-Ser catalytic triads associated with serine proteases and esterases.

ACKNOWLEDGMENTS

This work was supported by a grant from the Pearl and Aaron Forman Research Foundation (BTM), by National Institutes of Health Grant NS 29261 (AK) and by Grant

H-1190 from the Robert A. Welch Foundation (AK). We thank Dr. Mark Rogers of M-Scan for mass spectrometric analysis, Ms. Rosalinda Rodriguez and Mr. J. Steve Smith for expert technical assistance, and Angelina Mouton for preparation of the manuscript.

REFERENCES

1. Miller, B.T., Collins, T.J., Nagle, G.T., and Kurosky, A. (1992) J. Biol. Chem. 267, 5060-5069.
2. 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.
3. Kurosky, A., Miller, B.T., and Knock, S.L. (1993) J. Chromatogr. 631, 281-287.
4. Smith, J.S., Miller B.T., Knock S.L., and Kurosky, A. (1991) Anal. Biochem. 197, 247-253.
5. Smith, J.S., Miller, B.T., and Kurosky, A. (1991) Anal. Biochem. 197, 254-257.
6. Gounaris, A.D., and Perlmann, G.E. (1967) J. Biol. Chem. 242, 2739-2745.
7. Bodanszky, M., Fink, M.L., Klausner, Y.S., Natarajan, S., Tatemoto, K., Yiotakis, A.E., and Bodanszky, A. (1977) J. Org. Chem. 42, 149-152.
8. Walsh, C. (1979) Enzymatic Reaction Mechanisms, pp. 24-48, W.H. Freeman and Company, New York.
9. Bender, M.L., Bergeron, R.J., Komiyama, M. (1984) The Bioorganic Chemistry of Enzymatic Catalysis, pp. 116-158, John Wiley & Sons, New York.
10. Bodanszky, M. (1979) In The Peptides (E. Gross, and J. Meinhofer, Eds.), Vol. 1, pp. 139-195, Academic Press, New York.