A Carbon-13 Nuclear Magnetic Resonance Study of Hyperactive and Hypoactive Derivatives of Luteinizing Hormone-Releasing Hormone: des-Gly-NH_2^{10}-[D-Leu^6]LH-RH Ethylamide and des-Gly-NH_2^{10}-[L-Leu^6]LH-RH Ethylamide

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ABSTRACT: The carbon-13 (^{13}C) nuclear magnetic resonance (NMR) behavior of the hyperactive analogue of luteinizing-hormone-releasing hormone (LH-RH), des-Gly-NH_2^{10}-[D-Leu^6]LH-RH ethylamide, has been studied. The spectra are compared with those of the hypoactive des-Gly-NH_2^{10}-[L-Leu^6]LH-RH ethylamide. Spin–lattice relaxation times (T_1) of ^{13}C at natural abundance have been obtained for both peptides at 25 and 68 MHz in aqueous solution and used as monitors of flexibility at each carbon atom in the peptide backbone and side chains. The [D-Leu^6]- and [L-Leu^6]-substituted analogues show equal degrees of freedom within the peptide backbone and side chains. Substitution of the glycine amide moiety in position 10 by an ethylamide group causes an increase in flexibility of the backbone at that position. The increase in flexibility is not transmitted to the adjacent prolyl residue. The C-terminal peptide link at position 10 is proposed to restrict the flexibility of the prolyl residue in position 9, thus maintaining the motional characteristics of the LH-RH peptide backbone in the des-Gly-NH_2^{10}-[Leu^6]LH-RH ethylamide analogues. There were no significant differences in the ^{13}C chemical shifts or T_1 values between the hypoactive and hyperactive analogues in aqueous solution which would explain the differences in biological activity. Interaction between receptor and hormone may thus be required to induce, or select, the biologically active conformation of the hormone.

The synthesis of analogues of the hypothalamic peptide hormone, luteinizing hormone-releasing hormone (LH-RH), has led to compounds with high biological activities. Substitution of the C-terminal glycine amide residue of LH-RH (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2) with ethylamide resulted in a peptide with 300–500% the ovulation-inducing activity of LH-RH itself (Fujino et al., 1972). Replacement of the glycine at position 6 with an optically active amino acid resulted in dramatic changes in the biological activity. Substitution with amino acids of the D configuration generally enhances activity, whereas with L-amino acids the potency is greatly diminished (Monahan et al., 1973; Coy et al., 1974a,b; Fujino et al., 1974a,b). A further increase in hormonal activity was obtained when both a D-amino acid at position 6 and a C-terminal ethylamide were incorporated into a single molecule. Combination of an L-amino acid with the ethylamide modification leads to compounds less active than LH-RH. For example, des-Gly-NH_2^{10}-[D-Ala^6]LH-RH ethylamide has an ovulation inducing activity of 5000 to 8000% whereas des-Gly-NH_2^{10}-[L-Ala^6]LH-RH ethylamide has only 31% activity. Similar results are obtained with the corresponding leucine-containing analogues.

The hyperactivity of these D-amino acid containing analogues of LH-RH has been explained in terms of a restriction of the conformational freedom of LH-RH (Monahan et al., 1973). When a D-amino acid replaces glycine, a type-II β turn is stabilized. When an L residue is substituted for glycine, the β-II type bend is destabilized (Venkatachalam, 1968). However, it has recently been shown that a formal β-II type bend may not exist (Ling and Vale, 1975).

The difference in the biological activities between the D- and the L-amino acid analogues may be a consequence of changes in clearance rate, serum inactivation, or receptor affinity. The conformational, as well as structural, properties of peptide hormones can influence all these factors.

We have investigated the time-averaged conformational properties of LH-RH in aqueous solution using carbon-13 (^{13}C) nuclear magnetic resonance (NMR) spectroscopy (Deslauriers et al., 1975; Deslauriers and Somorjai, 1976). Spin–lattice relaxation times (T_1) of ^{13}C provide insight into the relative flexibility of the backbone and side chains of peptides and peptide hormones (Allerhand and Komoroski, 1973; Deslauriers et al., 1974; Deslauriers and Smith, 1976).

The purpose of the present study is to investigate the time-averaged conformational properties of the LH-RH analogues, des-Gly-NH_2^{10}-[D-Leu^6]LH-RH ethylamide (A-43818) and des-Gly-NH_2^{10}-[L-Leu^6]LH-RH ethylamide (A-46204). The former compound exhibits enhanced activity, when compared with LH-RH, in vivo in the ovulation-inducing assay and in vitro in the LH and FSH release assay (Fujino et al., 1974b). The [L-Leu^6] analogue exhibits decreased activity when compared with the parent compound.

Experimental Section

Material. Des-Gly-NH_2^{10}-[L-Leu^6]LH-RH ethylamide and des-Gly-NH_2^{10}-[D-Leu^6]LH-RH ethylamide were synthesized using solid phase or solution techniques (Fujino et al., 1974a; Arnold et al., 1973). Samples were studied at a con-

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‡ Abbreviations used: NMR, nuclear magnetic resonance; LH-RH, luteinizing hormone-releasing hormone; FSH, follicle stimulating hormone.

centration of 100 mg/ml in D_2O at a pH meter reading of 5.5. The pH was adjusted using CD_3COOH (Merck, Sharp and Dohme, Canada Ltd.).

Methods. Proton-decoupled ^{13}C NMR spectra were obtained at 25.16 MHz and 67.9 MHz. T_1 experiments were performed using the inversion-recovery method (Vold et al., 1968) with a ($T_\infty - 180° - \tau - 90°$) pulse sequence. T_1 values were calculated with an accuracy of ±15% from a least-squares fit to the best straight line on a semilogarithmic plot using

$$M(\tau) = M(0)(1-2\exp(-\tau/T_1))$$

$M(0)$ being the equilibrium value of the magnetization; for $\tau = 0$, $M = -M(0)$. $M(\tau)$ is the value of the magnetization at a given value of τ. Other experimental procedures are described in Deslauriers et al. (1975). The T_1 data reported at 67.9 MHz do not strictly follow the extreme narrowing condition and thus direct comparison of T_1 values obtained at the two fields should not be made. Preliminary data obtained at 25 MHz indicate that longer T_1 values can be qualitatively interpreted as indicating increased motion. A complete T_1 study was not performed at 25 MHz due to difficulties in obtaining adequate *S/N* ratios at this concentration.

Spectral Assignments. The ^{13}C NMR spectra of des-Gly-NH_2^{10}-[L-Leu^6]LH-RH ethylamide and des-Gly-NH_2^{10}-[D-Leu^6]LH-RH ethylamide were assigned by direct comparison with the spectra of LH-RH and des-Gly^{10}-LH-RH at the same pH (Deslauriers et al., 1975). Deletion of the glycyl residues in positions 10 and 6 produces few perturbations in the chemical shifts of the remaining residues. The resonances attributed to the glycyl residues disappeared and two new resonances attributed to the CH_2 and CH_3 groups of the ethylamide substituents were observed at 35.5 and 14.6 ppm downfield from external $(CH_3)_4Si$. The prolyl-9 chemical shifts are unaffected by the changes occurring at position 10. The leucyl-6 and -7 resonances are, for all practical purposes, degenerate at both NMR frequencies. The seryl and tyrosyl α-carbon resonances, which are degenerate in the [D-Leu^6] and [L-Leu^6] analogues, are at 56.8 and 56.3 ppm. Assignment of these resonances is not, however, possible and the numbers in Figure 1 may be reversed for these two α carbons. Thus the changes in chemical shifts are restricted to the immediate environment of the perturbation. All other chemical shifts are within 0.05 ppm of those reported for LH-RH at the same pH (Deslauriers et al., 1975).

Spin-Lattice Relaxation Times. The ^{13}C NT_1 values, where *N* is the number of hydrogens directly bonded to a carbon atom, measured at 68 MHz for des-Gly-NH_2^{10}-[D-Leu^6]LH-RH ethylamide and des-Gly-NH_2^{10}-[L-Leu^6]-LH-RH ethylamide are given in Figure 1. No values are reported for the imidazole carbons of histidine as these can partially exchange their protons for deuterons of the solvent, thereby rendering interpretation of such data complex. A more direct comparison of T_1 values is afforded by using the [D-Leu^6]- and [L-Leu^6]-substituted analogues rather than by comparing these with the parent compound, LH-RH. This method was chosen in view of the known influence of molecular weight and hydrophilicity of substituents on T_1 values (Komoroski and Levy, 1976). By using the D and L enantiomers for comparisons of T_1 values, it was judged that differences in conformational properties of the two peptides would be the primary cause of any differences in T_1 values. Although our stated accuracy of individual T_1 values is ±15%, smaller consistent trends would be significant. The T_1 values of the α carbons in the D and L derivatives were found not to differ significantly.

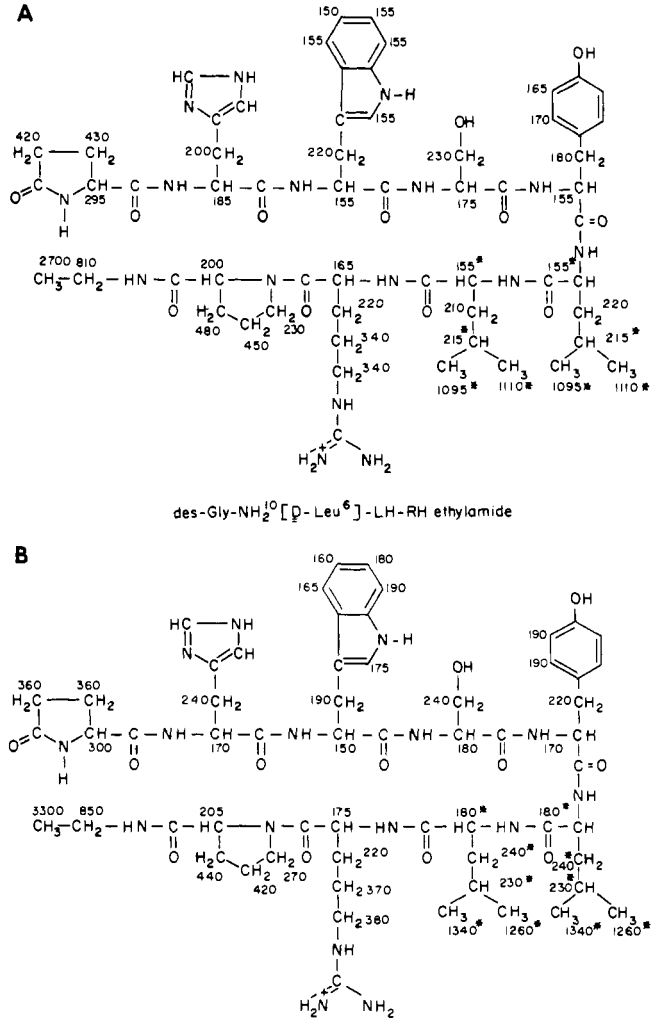

FIGURE 1: (A) Primary structure of des-Gly-NH_2^{10}-[D-Leu^6]LH-RH ethylamide showing NT_1 values (N is the number of protons directly bonded to a given carbon atom) in msec. Spectra were obtained at 68 MHz, 27 °C, 2000 scans/spectrum. Samples were 100 mg/ml; pH meter reading was 5.5. Asterisks indicate unresolved resonances. (B) Primary structure of des-Gly-NH_2^{10}-[L-Leu^6]LH-RH ethylamide showing NT_1 values. Other conditions are the same as in Figure 1A.

Discussion

Chemical Shifts. The ^{13}C chemical shifts of the [D-Leu^6]- and [L-Leu^6]-LH-RH analogues provide no evidence for differences in the average conformational constraints imposed on other residues of both compounds. Studies on cyclo(L-Pro-D-Leu) and cyclo(L-Pro-L-Leu) in D_2O have shown differences of 1.8 and 3.6 ppm for the α and β carbons of the leucyl residues. Differences of over 1.0 ppm were seen in the chemical shifts of the L-prolyl residue in these compounds (Deslauriers, Gzronka, and Walter, unpublished). The differences are attributed in part to the conformational changes which occur in the cyclic dipeptide as a consequence of the change in stereochemistry of the leucyl residue. The diketopiperazine ring is believed to change from the symmetric boat shape observed in cyclo(L-Pro-L-Leu) (Karle, 1972) to a more planar ring structure in cyclo(L-Pro-D-Leu). Studies on linear peptides containing the same sequence (L-Pro-L-Leu-Gly-NH_2 and L-Pro-D-Leu-Gly-NH_2), which are believed to be flexible in solution, did not show any differences in ^{13}C chemical shifts (Deslauriers and Walter, unpublished).

Thus it can be concluded that the similarity in ^{13}C chemical shifts between the [L-Leu^6]- and [D-Leu^6] analogues of LH-RH indicates that the *average* conformations of these molecules in solution are similar. The intrinsic flexibility of the peptide backbone in solution attenuates the consequences of the difference in stereochemistry between the two peptides.

Spin–Lattice Relaxation Times. The relative ratios of T_1 values within a given residue are similar in both the L and the D analogues. This indicates there is little change in the relative flexibility of the individual side chains. Furthermore the relative ratios of the T_1 values of the terminal and central α carbons are the same in both compounds, indicating no difference in relative mobilities of the chain termini in both compounds.

Although it is difficult to compare directly the observed T_1 values of the substituted LH-RH analogues with the parent compound, comparison of *relative* mobilities of various residues within each peptide is possible. In LH-RH the flexible backbone shows an increase in mobility mainly at the terminal and penultimate residues.

In LH-RH we also have observed that the glycyl residue in position 6 shows longer NT_1 values at all temperatures than do the nonterminal optically active residues. This was attributed to increased flexibility of the glycyl residue within the peptide backbone as a consequence of the absence of a bulky side chain (Deslauriers et al., 1975). These data have been further substantiated by studies on des-Gly-NH_2^{10}-LH-RH ethylamide in which removal of the glycyl residues in position 10 allows more accurate measurement of T_1 values for the remaining glycyl residue (Deslauriers, Komoroski, Levy, McGregor, Sarantakis, and Smith, unpublished). In contrast to the central glycyl residue of LH-RH, the D and L residues in position 6 of the analogues have T_1 values which are the same as those of the other optically active nonterminal residues. We do not observe any nonlinearity in the relaxation behavior of the degenerate resonances of the two leucyl residues which would have indicated the presence of two different relaxation times. This point will be verified by the study of analogues containing optically active residues not already present in the LH-RH sequence in order to avoid the overlap of resonances observed in the present analogues. The β and γ carbons of the prolyl and pyroglutamyl residues manifest greater flexibility than the α carbons, as in the parent hormone LH-RH.

A very significant change in the mobility of the LH-RH peptide backbone occurs at position 10 when the glycine amide moiety is replaced by ethylamide. In LH-RH the ratio of NT_1 values between the α-carbons of the glycyl-10 and prolyl-9 residues is ca. 2 whereas the difference in NT_1 values at the corresponding positions in the des-Gly-NH_2^{10}-[Leu^6]LH-RH ethylamide derivatives is 4. The increased ratio is indicative of a greater mobility for the *N*-ethyl methylene groups of the two analogues than for the glycine methylene group of LH-RH. This is not surprising in view of the differences in formula weights of the substituents attached to the CH_2 groups. In LH-RH the amide substituent is considerably bulkier than the methyl substituent of the ethylamide derivative. Furthermore, the amide group can interact with the aqueous environment by hydrogen-bonding, whereas the ethyl moiety cannot. A more surprising observation is that the perturbation at position 10 is *not* transmitted through the peptide link to the prolyl residue at position 9. The ratio of T_1 values between the α carbons of prolyl-9 and arginyl-8 is 1.2–1.3 in LH-RH and in the *N*-ethylamide derivatives. Thus the peptide link and the prolyl residue effectively anchor the peptide backbone in solution. The effectiveness of the peptide link in defining the conformational average may be a significant factor in the conformational stability of smaller peptide hormones in solution. For small peptides in solution, such as thyrotropin-releasing hormone (<Glu-His-Pro-NH_2) and melanocyte-stimulating hormone release-inhibiting factor (Pro-Leu-Gly-NH_2) the presence of the C-terminal amide group can decrease the flexibility of the C-terminal residue and facilitate the appropriate positioning of all the amino acids in families of conformations which will interact more readily with the hormone receptor. Thus we see that in the analogues studied here the average conformational characteristics of the peptide backbone from position 9 toward the N-terminus are maintained after deletion of the glycyl residue in position 10. The change in position 6 restricts the motion of the backbone at that specific position. The increase or decrease in activity of these analogues, compared with LH-RH, may partially reflect the steric accessibility of the active receptor-bound conformation.

Conclusion

These studies have compared the conformational flexibilities, as monitored by the ^{13}C NMR behavior, of two analogues of LH-RH. The two analogues which differ in positions 6 and 10 from the naturally occurring LH-RH peptide show enhanced and decreased LH-RH activities. Substitution of glycine-6 by D- or L-leucine does not perturb the ^{13}C spectrum of the adjacent residues, indicating the replacement does not cause steric strain of any other residues in the peptide. The relative mobilities of the carbons in the side chains of the LH-RH analogues and LH-RH itself are similar. Substitution of the glycyl residue in position 10 by ethylamide produces a very marked, yet localized, perturbation of the mobility of the C-terminal end of the peptide. The perturbation is not transmitted to the prolyl residue in position 9 nor to any other residue. The change at position-10 does not perturb the time-averaged conformation of the peptide backbone. The substitution of an optically active residue for glycine in position 6 restricts the mobility of the α carbon at that position. This point is being further investigated using D,L pairs of amino acids which facilitate the monitoring of backbone mobility at position 6 by allowing greater dispersion of the C-13 resonances.

The conformational differences between LH-RH and the analogues studied herein are revealed mainly by the flexibility of the C-terminal moiety and at position 6 on the backbone. The conformational equilibrium of the peptide backbone which exists in solution, as monitored by the ^{13}C T_1 values, is not greatly perturbed by the modifications in positions 6 and 10. However, these compounds do exhibit widely varying degrees of biological activity. It can be envisaged that the conformation of the receptor bound peptide, as well as the conformation required to bind to degradative enzymes, is determined to a certain degree by the geometry of the receptor itself. The presence of a β turn in LH-RH and LH-RH analogues may be necessary for receptor interaction, but it may be induced by binding to the receptor. The latter is facilitated by the flexibility of the peptide backbone in LH-RH and its analogues.

Further study of conformational properties of LH-RH analogues in solution will involve temperature and solvent perturbations (Urry et al., 1975) which can provide insight into the relative populations of preferred conformers in the D- and L-substituted peptides. These studies provide a basis for the investigation of the conformational characteristics of LH-RH and LH-RH analogues bound to receptors. Enrichment of

LH-RH and its analogues in ^{13}C should allow investigation of the binding properties of these peptides, if not to membrane receptors due to low receptor populations, to model receptors.

References

Allerhand, A., and Komoroski, R. A. (1973), *J. Am. Chem. Soc. 95,* 8228–8231.

Arnold, W. H., White, W., and Fluoret, G. (1973), *J. Med. Chem. 16,* 1054–1055.

Coy, D. H., Coy, E. J., Schally, A. V., Vilchez-Martinez, J. A., Debeljuk, L., Carter, W. H., and Arimura, A. (1974a), *Biochemistry 13,* 323–326.

Coy, D. H., Coy, E. J., Schally, A. V., Vilchez-Martinez, J., Hirotsu, Y., and Arimura, A. (1974b), *Biochem. Biophys. Res. Commun. 57,* 335–340.

Deslauriers, R., Levy, G. C., McGregor, W. H., Sarantakis, D., and Smith, I. C. P. (1975), *Biochemistry 14,* 4335–4343.

Deslauriers, R., and Smith, I. C. P. (1976), in Topics in Carbon-13 NMR Spectroscopy, Vol. 2, Levy, G. C., Ed., New York, N.Y., Wiley-Interscience, Chapter 1, pp 1–80.

Deslauriers, R., Smith, I. C. P., and Walter, R. (1974), *J. Am. Chem. Soc. 96,* 2289–2291.

Deslauriers, R., and Somorjai, R. (1976), *J. Am. Chem. Soc.* (in press).

Fujino, M. Fukuda, T., Shinagawa, S., Obayashi, M., Shinagawa, S., Fukuda, T., Kitada, C., Nakayama, R., Yamakazi, I., White, W. F., and Rippel, R. H. (1972), *Biochem. Biophys. Res. Commun. 49,* 863–869.

Fujino, M., Fukuda, T., Shinagawa, S., Kobayashi, S., Yamazaki, I., Nakayama, R., Seeley, J. H., White, W. F., and Rippel, R. H. (1974b), *Biochem. Biophys. Res. Commun. 60,* 406–413.

Fujino, M., Yamazaki, I., Kobayashi, S., Fukuda, T., Shinagawa, S., Nakayama, R., White, W. F., and Rippel, R. H. (1974a), *Biochem. Biophys. Res. Commun. 57,* 1248–1256.

Karle, I. L. (1972), *J. Am. Chem. Soc. 94,* 81–84.

Komoroski, R. A., and Levy, G. C. (1976), unpublished results.

Ling, N., and Vale, W. (1975), *Biochem. Biophys. Res. Commun. 63,* 801–806.

Monahan, M. W., Amoss, M. S., Anderson, H. A., and Vale, W. (1973), *Biochemistry 12,* 4616–4620.

Urry, D. W., Ohnishi, T., Long, M. M., and Mitchell, L. W. (1975), *Int. J. Pept. Protein Res. 7,* 367–378.

Venkatachalam, C. M. (1968), *Biopolymers 6,* 1425–1436.

Vold, R. L., Waugh, J. S., Klein, M. P., and Phelps, D. E. (1968), *J. Chem. Phys. 48,* 3831–3832.

Superactivation of Neutral Proteases: Acylation with *N*-Hydroxysuccinimide Esters†

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ABSTRACT: A series of *N*-hydroxysuccinimide esters of acylamino acids previously shown to acylate and thereby increase the activity of thermolysin by several orders of magnitude (Blumberg, S., and Vallee, B. L. (1975), *Biochemistry 14,* 2410) has been used to modify the related neutral proteases from *Bacillus subtilis, Bacillus megaterium,* and *Aeromonas proteolytica.* Each of these enzymes is activated to a level characteristic of the particular protein and the particular acyl group incorporated when monitored with the substrate furylacryloyl-Gly-Leu-NH_2. Thus, for the modification of *B. megaterium, B. subtilis,* and *A. proteolytica* proteases with Ac-Trp-ONSu, k_{cat}/K_m increases 11-, 2.5-, and 18-fold whereas those of the Ac-Phe(4-DnpNH)-ONSu derivatives increase 23-, 22-, and 3.4-fold, respectively. Absorption spectra of the Ac-Phe(4-DnpNH)-ONSu modified enzymes before and after deacylation with hydroxylamine indicate that from 1 to 2 residues are modified. The rate of removal of the Ac-Phe(4-DnpNH) label by 0.1 M hydroxylamine correlates directly with that of the return of native enzymatic activity, at a rate comparable with the rate of deacylation of *O*-acyltyrosine models. The competitive inhibitors Zn^{2+} and β-phenylpropionyl-Phe do not prevent activation indicating that modification occurs at a site(s) distinct from that at which inhibitors bind. The degree of activation depends also on the substrate employed, generally being greater for substrates which the native enzymes hydrolyze slowly. These data are interpreted to indicate the modification of a residue near the active site, but which serves as a subsite for substrate interaction.

Complementary interactions of enzyme active centers with substrates and inhibitors are fundamental to the mode of action of enzymes (cf. Jencks, 1975). Synthetic approaches designed to further the understanding of enzyme mechanisms, however, have been restricted largely to systematic variations of substrate and inhibitor structure. Chemical modification resulting in incorporation of organic moieties into the active center of enzymes augments this approach when modulating enzymatic function. We have recently demonstrated that *N*-hydroxysuccinimide esters of acylamino acids or peptides covalently link amino acids or peptides to a chemically reactive side chain of thermolysin, thereby markedly increasing its activity (Blumberg et al., 1973, 1974; Blumberg and Holmquist, 1973; Blumberg and Vallee, 1975). The structure of these reagents

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