Conformational Studies of Human Des-Trp[1],Nle[12]-minigastrin in Water–Trifluoroethanol Mixtures by ^{1}H NMR and Circular Dichroism†

Stefano Mammi, Nancy J. Mammi, and Evaristo Peggion*

Biopolymer Research Center, Department of Organic Chemistry, University of Padova, Via Marzolo 1, 35131 Padova, Italy

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ABSTRACT: The ^{1}H NMR spectrum of the title peptide, H-Leu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH$_2$, in 90% H_2O/10% D_2O was assigned by two-dimensional methods, and the displacement of the proton resonances upon addition of 2,2,2-trifluoroethanol (TFE) was followed. This permitted the assignment of the spectrum in 90% TFE/10% D_2O. While the water conformation of the minigastrin analogue is random, the CD spectrum indicates that an ordered structure is present in TFE. Variable-temperature NMR data in this medium show that six amide protons have low temperature coefficients, two of the five Glu's, Trp, Nle, Asp, and Phe. These results were interpreted in terms of an α-helical stretch comprising the Leu and the five Glu residues and a 3_{10}-helix initiated by a β-turn at the sequence -Ala-Tyr-Gly-Trp-. Both CD and NMR data at different solvent compositions show two regions of conformational change, between 20 and 25% water and above 60% water.

It is well established that the C-terminal tetrapeptide amide, -Trp-Met-Asp-Phe-NH$_2$, is essential for biological activity of the gastrin hormones (Tracy & Gregory, 1964). The role of the N-terminal portion has been investigated by Göhring et al. (1984), who found that the biological potencies of gastrin fragments increase with increasing chain length. Specifically, in a study of six gastrin analogues elongated stepwise from the octapeptide, pGlu-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH$_2$, to the tridecapeptide, H-Leu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Nle-Asp-Phe-NH$_2$ (des-Trp[1],Nle[12]-minigastrin), the physiological potency increased from approximately 20% to 90% that of little gastrin [pGlu-Gly-Pro-Trp-Leu-(Glu)$_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH$_2$]. [The replacement of Met with Nle has been shown to have no effect on biological activity by Morley et al. (1964).] The conformation of the gastrin hormones in H_2O was shown to be random by circular dichroism (CD) spectra (Peggion et al., 1981). The NMR results of Torda et al. (1985) further indicate little or no secondary structure of little gastrin in aqueous solution. Thus, if an "active structure" of the gastrin hormone exists, it should be formed in the lipophilic environment at the receptor binding site. In a CD study on the conformation of the gastrin fragments described above, we found that the degree of secondary structure in trifluoroethanol (TFE) solutions correlates well with biological potency (Peggion & Foffani, 1985; Peggion et al., 1985a). Specifically, the increase of ordered conformation in TFE and the increase of biological activity upon chain elongation match the same profile. This observation led to the hypothesis that the conformation assumed by gastrin hormones in TFE is of biological importance. This hypothesis was further supported by the observation that both little gastrin and des-Trp[1],Nle[12]-minigastrin adopt a similar conformation in TFE and in water solutions containing sodium dodecyl sulfate micelles (Wu et al., 1982; Mammi et al., 1987).

On the basis of CD results and sequence analysis according to Chou and Fasman (1977), a conformational model was proposed for the gastrin peptides in TFE (Peggion et al., 1985a). According to this model, the structure of minigastrin is characterized by an α-helical segment at the N-terminus, comprising the sequence of five glutamic acid residues, and by a β-turn in the central part of the molecule, possibly located in the sequence Ala-Tyr-Gly-Trp.

According to theoretical conformational energy calculations by Abillon et al. (1981), the structure of the biologically important sequence at the C-terminus might involve a C_7-turn, with a hydrogen bond from Phe-NH to Nle-CO.

To test our conformational hypothesis, an unambiguous determination of the pattern of intramolecular hydrogen bonds is required. In a previous investigation, we reported the 400-MHz proton NMR spectrum in the amide and aromatic region of des-Trp[1],Nle[12]-minigastrin in 90% TFE/10% water (v/v) (Mammi et al., 1986). A number of amide resonances were well resolved, but their assignment was not made because of severe difficulties caused by solvent peaks. We found, however, that at least four amide resonances had low temperature coefficients, indicating the presence of intramolecular hydrogen bonds.

In the present paper we report the results of NMR and CD studies of des-Trp[1],Nle[12]-minigastrin in TFE–H_2O mixtures of various composition. Resonance peaks were assigned and the amide groups involved in intramolecular hydrogen bonds identified. These data allow a more complete description of the conformational features of the hormone.

Materials and Methods

Des-Trp[1],Nle[12]-minigastrin was provided by Prof. E. Wünsch of the Max-Planck Institut für Biochemie, Münich (Göring et al., 1984). 2,2,2-Trifluoroethanol (TFE) was purchased from Merck. Proton NMR spectra were obtained with a Bruker AM 400 instrument equipped with an Aspect 3000 computer and operating at 400 MHz. Peak positions were measured relative to tetramethylsilane (TMS) as internal standard. Samples for NMR were prepared in the range of 3–5 mM peptide with 15 mM NH_4OH to increase solubility. Water samples were prepared with 90% H_2O and 10% D_2O or 100% D_2O. The pH was adjusted to 6.5 with HCl. TFE

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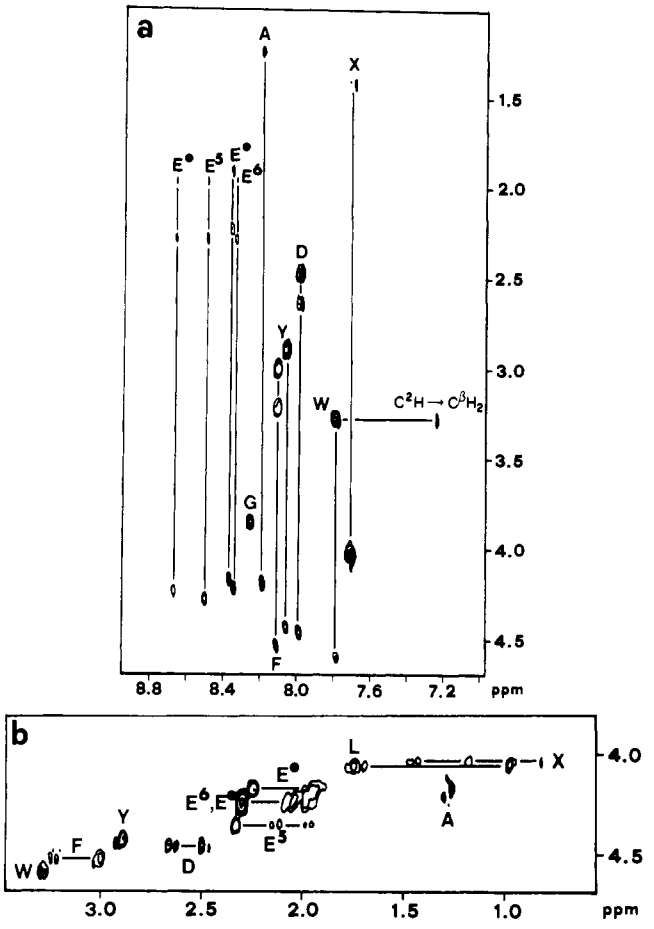

FIGURE 1: Two-dimensional homonuclear Hartmann–Hahn spectrum of des-Trp[1],Nle[12]-minigastrin (4.43 mM) in 90% H_2O/10% D_2O: (a) aliphatic (F1 axis)-NH and aromatic (F2 axis), and (b) $C^{\alpha}H$ (F1 axis)-aliphatic (F2 axis) regions. Composite pulse of the spin lock was cycled 16 times, and the spin-locking pulses were 3 ms long for a total mixing time of 87 ms. The spin-locking power was $\gamma B_2/2\pi \approx 3.25$ kHz. The spectral width in both dimensions was 4350 Hz. To give a final spectrum of 2K by 2K data points, 512 FID's of 64 scans each were collected, and zero-filling was carried out in the t_1 dimension. Gaussian multiplication was applied in both dimensions prior to transformation. Only positive peaks are plotted of the pure absorption mode spectrum obtained by using the TPPI method (Marion & Wüthrich, 1983). Residues are labeled by using the one-letter code, and X stands for Nle. (●) Glu[3] and Glu[4] cannot be distinguished.

samples contained a minimum of 5% D_2O in which the peptide was dissolved before addition of the TFE.

For peak assignments in 90% H_2O/10% D_2O, the two-dimensional homonuclear Hartmann–Hahn experiment as described by Bax and Davis (1985) was employed with 1.5-s presaturation of the H_2O peak. The 2D Hartmann–Hahn spectrum reveals J connectivities of entire spin systems. The pulse sequence used is presaturation–$90°_x$–t_1–spin lock–t_2.

The spin lock consists of two parts. A trim pulse along the x axis at the beginning and the end of the spin-locking period maintains x magnetization and dephases all other magnetization. Between these two pulses is a series of pulses of the form (ABBA BBAA BAAB AABB $180°_x)_n$, where A and B rotate x magnetization through the z axis with three pulses each, A = $90°_{-y}$–$180°_x$–$90°_y$ and B = $90°_y$–$180°_{-x}$–$90°_y$. When n is an even integer, the 180° pulse refocuses x magnetization, correcting for small pulse imperfections.

One-dimensional nuclear Overhauser enhanced (NOE) (Noggle & Schirmer, 1971) experiments were used to clarify assignment ambiguities by using a 100% D_2O sample of the gastrin peptide.

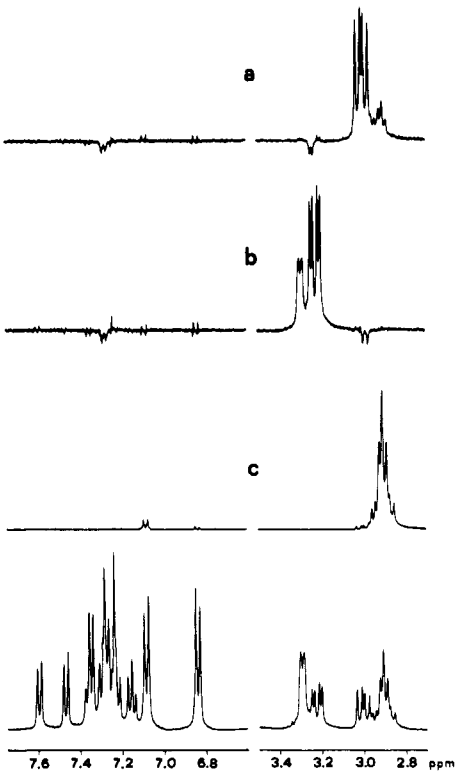

FIGURE 2: One-dimensional NOE difference spectra of des-Trp[1],Nle[12]-minigastrin 4.42 mM in D_2O with (a) one Phe $C^{\beta}H$ irradiated, (b) the other Phe $C^{\beta}H$ irradiated, and (c) the Tyr $C^{\beta}H_2$ irradiated. Each peak of the saturated multiplet was irradiated in turn for 50 ms with a power of 0.2 W attenuated by 35 dB. The cycle was repeated to obtain a total irradiation time of 5 s.

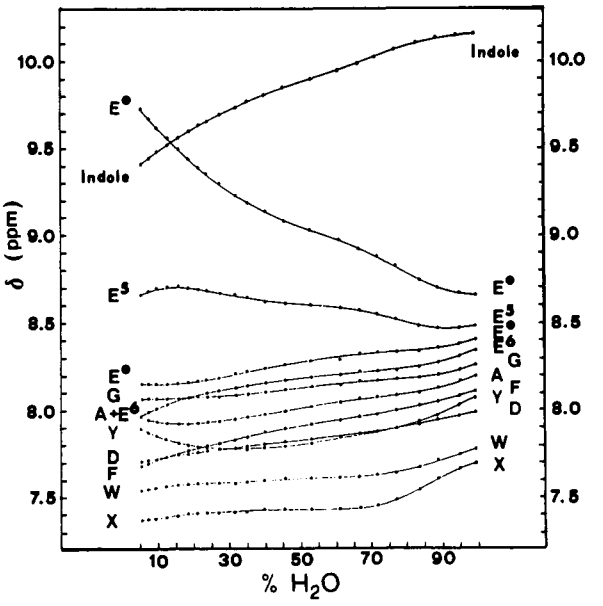

FIGURE 3: Chemical shifts versus solvent composition of the amide resonances of des-Trp[1],Nle[12]-minigastrin in H_2O–TFE mixtures. Residues are labeled by using the one-letter code, and X stands for Nle. (●) Glu[3] and Glu[4] cannot be distinguished.

Peak assignments in TFE were obtained from H_2O–TFE titration and by the two-dimensional Hartmann–Hahn experiment with 5-s multiple peak presaturation to suppress the TFE quartet.

During the water–TFE titration, it was possible to obtain clear spectra suppressing only the TFE methylene quartet by using multiple peak saturation for H_2O concentrations of less than 40%. Above 60% H_2O, the $1\bar{3}3\bar{1}$ pulse sequence (Hore,

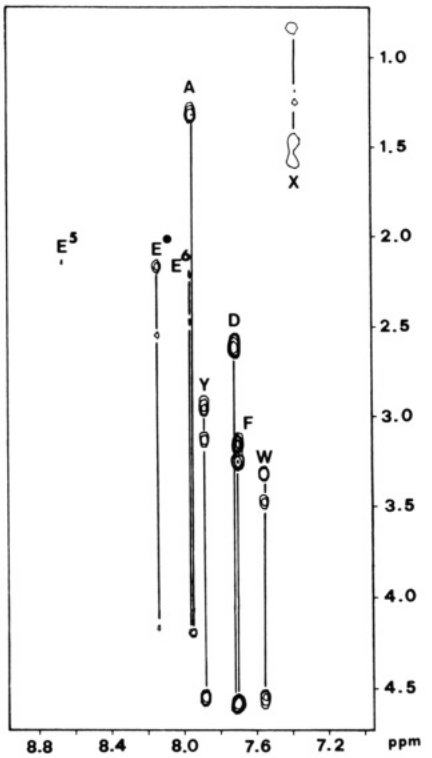

FIGURE 4: Aliphatic (F1 axis)-NH and aromatic (F2 axis) region of the two-dimensional homonuclear Hartmann–Hahn spectrum of des-Trp[1],Nle[12]-minigastrin (≈4.0 mM) in 94% TFE/6% D_2O. Experimental conditions were as described in Figure 1, except for the spectral width (4505 Hz) and the final size of the data point matrix (1K by 1K). Residues are labeled by using the one-letter code, and X stands for Nle. (●) Glu[3] and Glu[4] cannot be distinguished.

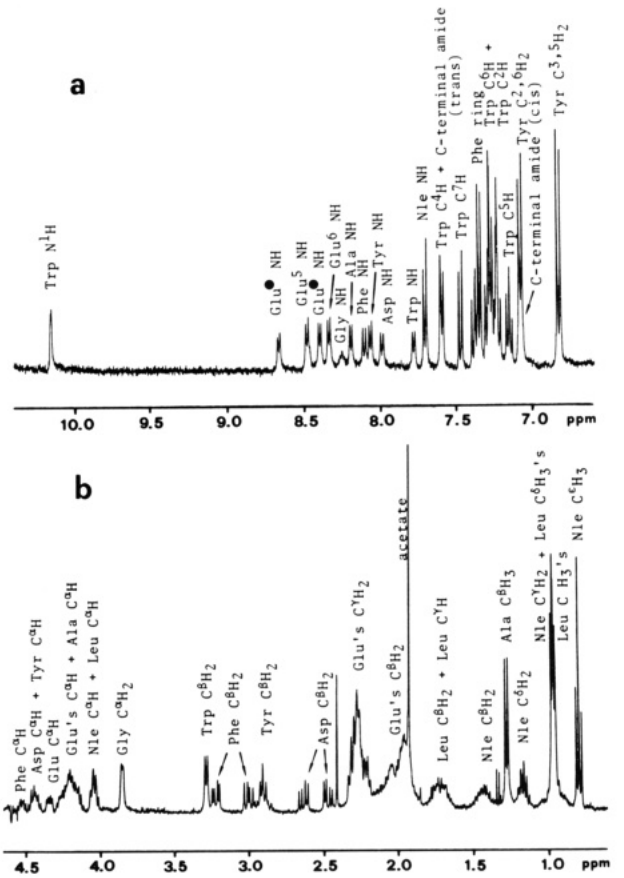

FIGURE 5: Assigned 400-MHz spectrum of des-Trp[1],Nle[12]-minigastrin in 90% H_2O/10% D_2O: (a) downfield region and (b) upfield region. (●) Glu[3] and Glu[4] cannot be distinguished.

1983) was used to eliminate the H_2O peak. From 40 to 60% H_2O, both multiple peak presaturation of the TFE quartet and the $1\bar{3}3\bar{1}$ sequence were used.

CD spectra were obtained on a Jasco Model J-500 automatic recording spectropolarimeter equipped with a Jasco DP-501 N data processor. Spectra were obtained with a 2-nm bandwidth, a scan speed of 10 nm/min, and a time constant of 8 s. The signal-to-noise ratio was improved by accumulating 16–64 scans. To eliminate errors from instrumental drift, the base line in air was recorded and subtracted after each scan. For the TFE–H_2O titration, the des-Trp[1],Nle[12]-minigastrin concentration was 0.01–0.03 mM. A quartz cell of 0.1-cm path length was used. Spectra are reported in terms of molar ellipticity units (deg cm^2 $dmol^{-1}$).

RESULTS

Proton NMR Studies in Water–TFE Mixtures. For adequate solubility of des-Trp[1],Nle[12]-minigastrin in both water and TFE–water solutions, it was necessary to add NH_4OH (15 mM). Because of the tendency to aggregate already encountered in the past (Peggion et al., 1985a,b), particular care was used to prepare the TFE solutions for NMR. The CD spectrum of all the NMR samples was measured and found to be identical with those recorded at sample concentrations of 0.01 mM, indicating that aggregation did not occur.

The assignment of des-Trp[1],Nle[12]-minigastrin in 90% TFE proved to be extremely difficult by the usual methods because of the large OH peak and the methylene quartet of the solvent. Deuteriated TFE was not used because of the labile NH protons and because we have been unable to find TFE-d_3 of acceptable purity.

To facilitate resonance peak assignment, we chose to assign the water spectrum and follow the resonance peaks from H_2O to TFE. From the 2D Hartmann–Hahn spectrum (Figure 1), the H_2O spectrum was nearly fully assigned. The Trp resonances were distinguishable from those of Tyr and Phe by a cross-peak showing the long-range coupling between the Trp C^2 aromatic proton and the C^β protons at 3.28 ppm. The resonances of $C^\gamma H_2$ and $C^\delta H_2$ protons of Nle were identified from a COSY spectrum (Bax, 1981, not shown). The following ambiguities remained: (1) the NH, $C^\alpha H$, and $C^\beta H_2$ peaks of the Phe and Tyr residues could not be distinguished; (2) only four of the five glutamic NH peaks were visible; and (3) the four Glu amide protons could not be distinguished one from another.

Using a 100% D_2O sample of the minigastrin analogue, we

Table I: Temperature Coefficients (Δ ppb/K) of Amide Protons of Des-Trp[1],Nle[12]-minigastrin in TFE–H_2O Mixtures

	% H_2O (v/v)			
residue	10	31	72	100
Glu[2]	broad	broad	broad	broad
Glu[a]	−8.9 ± 1.4	−7.2 ± 0.2	−7.4 ± 0.2	−6.6 ± 1.4
Glu[a]	−4.5 ± 0.9	−4.0 ± 0.3	−4.8 ± 0.1	−6.5 ± 0.9
Glu[5]	+0.9 ± 1.3	−4.8 ± 0.3	−6.5 ± 0.3	−6.6 ± 1.0
Glu[6]	−0.7 ± 0.8	−0.9 ± 0.3	−2.4 ± 0.3	−5.9 ± 0.7
Ala	−4.8 ± 0.9	−4.8 ± 0.3	−5.3 ± 0.1	−7.5 ± 1.2
Tyr	−7.1 ± 0.7	−5.6 ± 0.5	−5.4 ± 0.1	−7.9 ± 1.4
Gly	−5.3 ± 0.7	−5.4 ± 0.3	−5.4 ± 0.4	−5.6 ± 1.5
Trp	−2.3 ± 0.6	−2.3 ± 1.0	−2.5 ± 0.0	−4.7 ± 0.4
Nle	−1.6 ± 0.8	*b*	−1.6 ± 0.2	−3.9 ± 0.2
Asp	−1.8 ± 0.9	−3.0 ± 0.2	−3.6 ± 0.0	−4.0 ± 0.5
Phe	−3.0 ± 0.7	−3.8 ± 0.2	−5.1 ± 0.4	−6.7 ± 0.9

[a] Glu[3] and Glu[4] cannot be distinguished. [b] The temperature coefficient could not be calculated because of overlapping peaks. 95% confidence intervals are reported.

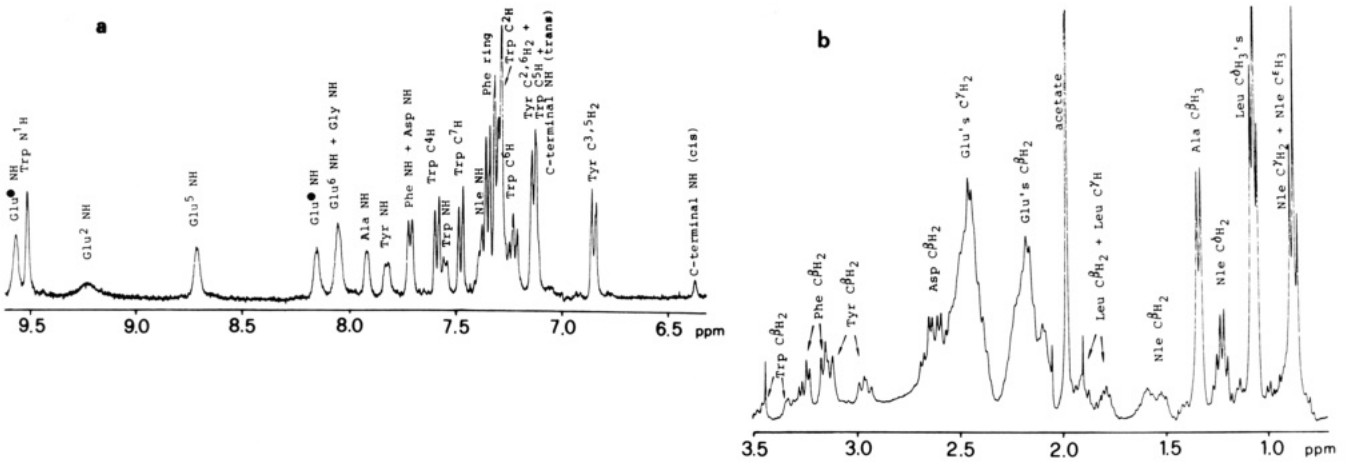

FIGURE 6: Assigned 400-MHz spectrum of des-Trp[1],Nle[12]-minigastrin in 90% TFE/10% D_2O: (a) downfield region and (b) upfield region. (●) Glu^3 and Glu^4 cannot be distinguished.

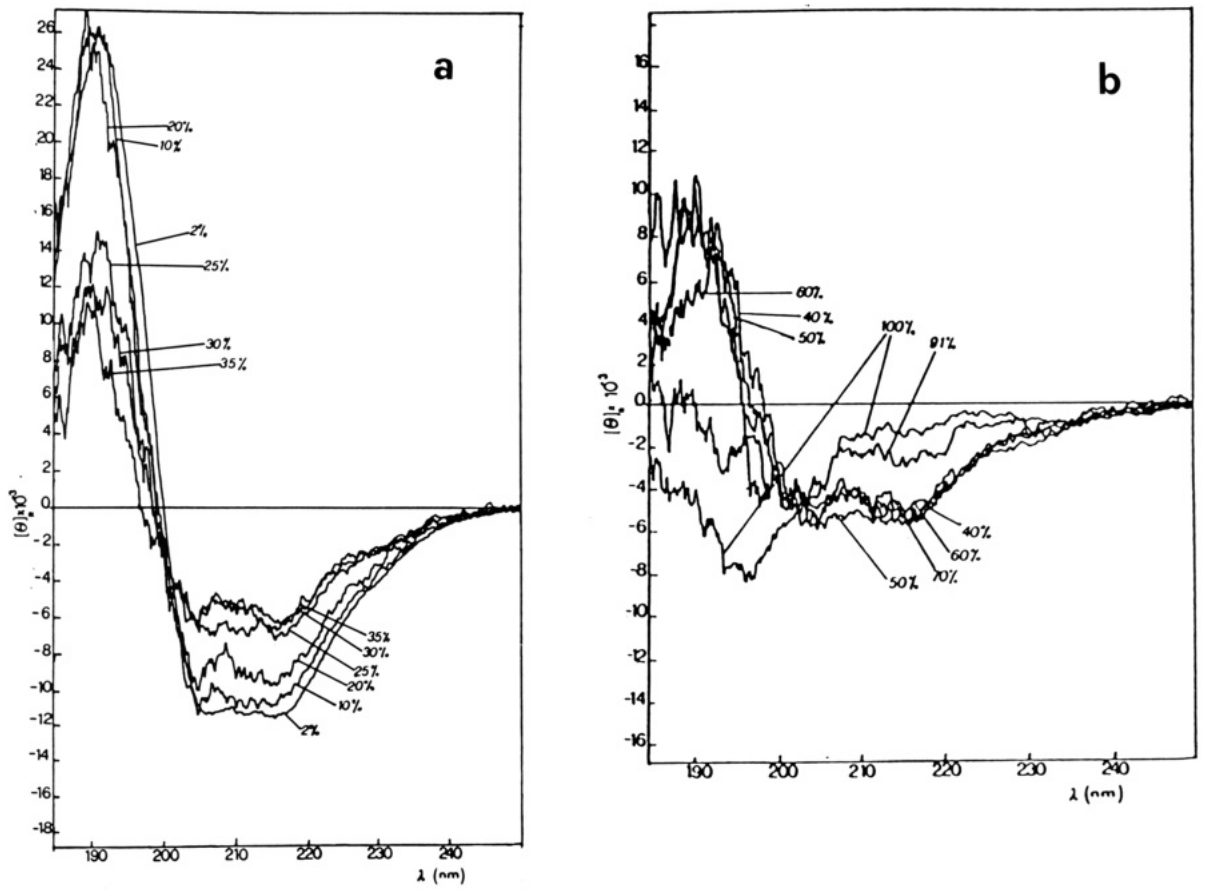

FIGURE 7: CD spectra of des-Trp[1],Nle[12]-minigastrin in various mixtures of H_2O and TFE. The percentage of H_2O is indicated (a) from 2 to 35% and (b) from 40 to 100%.

obtained one-dimensional NOE spectra to distinguish Tyr and Phe resonances. Irradiation of the $C^{\beta}H$ multiplets gave NOE's to aromatic resonances as shown in Figure 2. It is noteworthy that the Phe side chain is in the fast motion limit giving positive NOE's and the Tyr side chain is in the slow motion limit giving negative NOE's. Thus, the overall tumbling of the peptide is slow, but the Phe side chain is free to move at a higher frequency.

Difficulties in the assignment of the individual Glu NH resonance peaks remain. The first Glu-NH in the sequence could be expected to have a very broad resonance peak, as is frequently observed for the second residue in a peptide when the N-terminus is a free amine. We have observed a very broad NH peak at 9.2 ppm at concentrations of 75–90% TFE probably due to this residue. The α-helical structure that is highly probable in the region of the five glutamic residues would require the amide protons of Glu residues in position five and six to be hydrogen bonded. Thus, the two Glu-NH's with the lowest temperature coefficients in TFE (see below), which give resonance peaks at 8.48 and 8.34 ppm in the water spectrum, were assigned to Glu^5 and Glu^6, respectively. The distinction between these two resonances was made on the basis of their temperature coefficients at various solvent compositions (see below). One hydrogen bond is broken at 31% water, while the other is stable to 72% water. The former was attributed to the residue closer to the chain end, i.e., Glu^5. The remaining Glu amide protons are attributed to Glu^3 and Glu^4 and cannot be distinguished one from another.

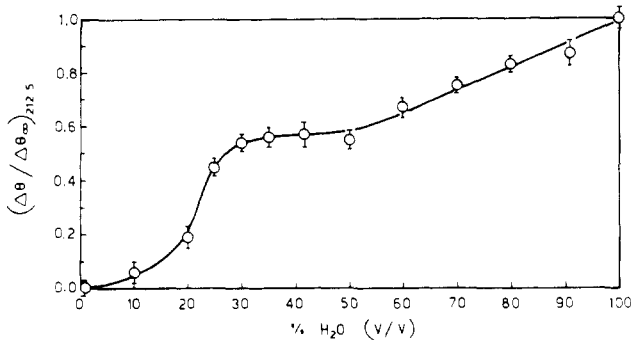

FIGURE 8: Relative variation of the molar ellipticity of des-Trp[1],Nle[12]-minigastrin versus solvent composition in H_2O–TFE mixtures.

During the water–TFE titration (Figure 3), we were able to follow all of the amide resonances except through the intersection of Tyr, Asp, and Phe at approximately 20% H_2O. These ambiguities were clarified by a two-dimensional homonuclear Hartmann–Hahn experiment with multiple peak saturation of the TFE quartet on a solution of 94% TFE. In contrast to previous attempts at COSY (Bax, 1981) and 2D relayed coherence transfer (Eich et al., 1982), the Hartmann–Hahn experiment showed numerous connectivities that confirmed our previous assignments. Further, we were able to distinguish the resonance peaks of Asp, Tyr, and Phe (Figure 4). The assigned one-dimensional spectra of the minigastrin analogue in H_2O and 90% TFE are shown in Figures 5 and 6.

Temperature studies were run on four samples during the titration, at 10%, 31%, 72%, and 100% (v/v) water, to identify hydrogen-bonded amide protons. Temperature coefficients of the amide protons are shown in Table I. We considered solvent-shielded amide protons those with temperature coefficients not exceeding −3.0 ppb/K.

CD Studies in Water–TFE Mixtures. In order to check the conformational state of the hormone during the NMR titration, CD measurements were carried out in water–TFE mixtures of various composition. Addition of water causes a progressive destruction of the ordered conformation of des-Trp[1],Nle[12]-minigastrin (Figure 7). The intensity of the characteristic double-negative maximum CD spectrum decreases as the water content is increased. The profile of the relative variation of molar ellipticity at fixed wavelength as a function of the solvent composition shows two conformational changes (Figure 8). The initial structure remains stable up to a water content of about 15%. Between 20% and 25% water there is a sharp, cooperative change to a structure that remains stable up to about 50–60% water. Further increase of the water content induces a noncooperative change to a random conformation.

Discussion

The assignment of proton resonances and temperature-dependence studies of des-Trp[1],Nle[12]-minigastrin in TFE permitted the identification of the amide protons involved in intramolecular hydrogen bonds. As stated in the introduction, both CD studies and sequence analysis according to Chou and Fasman (1977) indicate the presence of an α-helical segment at the N-terminus. The occurrence of two hydrogen-bonded amide protons of Glu residues provides support for this hypothesis (Table I). These Glu amides were assumed to be of Glu[5] and Glu[6], hydrogen bonded to Leu[1]-CO and Glu[2]-CO, respectively.

To assess the conformation of the remainder of the molecule in light of the four hydrogen-bonded amides of Trp, Nle, Asp, and Phe, we consider several possible structures. Since the only helix-breaking residue in the sequence is Gly, an α-helix could extend throughout the molecule, with the Gly[9] causing only a local disruption. With this interpretation, however, it is difficult to explain why Ala and Tyr amide protons are solvent exposed. We conclude that the α-helical segment ends with the Glu[6] residue.

Conformational energy calculations on the C-terminal tetrapeptide amide showed that structures containing one or two C_7 rings were preferred over an α-helix (Abillon et al., 1981). These conformations, which include hydrogen bonding of Phe-NH to Nle-CO and of Asp-NH to Trp-CO, should give rise to a CD band at about 230 nm (Rose et al., 1985). Such a band has not been identified in the CD pattern of the minigastrin analogue.

The sequence Ala-Tyr-Gly-Trp may form a β-turn, according to our previous CD results and Chou and Fasman prediction. The amide proton of Trp would be hydrogen bonded to Ala-CO. We do find that Trp-NH is hydrogen bonded and conclude that the hypothesis of a β-turn in this position is the most acceptable.

If Trp-NH is involved in a C_{10} structure, the following residue can assume an α-helical conformation only if Nle-NH is hydrogen bonded to Ala-CO, which is already involved in a hydrogen bond to Trp-NH. The presence of this bifurcated hydrogen bond cannot be ruled out by our results. A regular α-helix starting from the Trp residue, involving a hydrogen bond between Trp-NH and Glu[6]-CO, could also exist. In this case, however, it is difficult to understand why the first helical segment ends at Glu[6].

A conformation that accounts for the hydrogen bonds in the C-terminal region and is consistent with the CD pattern comprises a series of type I or type III β-turns with hydrogen bonds of Nle-NH to Tyr-CO, Asp-NH to Gly-CO, and Phe-NH to Nle-CO. This is equivalent to a segment of 3_{10}-helix starting with Ala-CO and including the six C-terminal residues. The proposed hydrogen-bond pattern is shown in Figure 9.

This model accounts for the experimental observations of previous CD studies and the NMR data presented here, but must still be considered as tentative. We have not identified the proton acceptors in the hydrogen-bond system. Further, we have not been able to obtain NOE's for the hormone in TFE. These data could provide a clearer description of the hormone conformation. In this context, the possibility of involvement of the carboxylate side chains of the Glu residues in hydrogen bonding cannot be ruled out a priori.

In the proposed structure, the Gly residue appears to play a crucial role in determining the conformation of minigastrin. Replacement of Gly with an α-helix-forming residue would

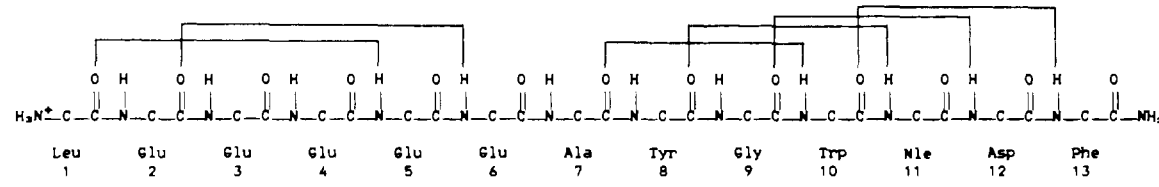

FIGURE 9: Hydrogen-bonding pattern of the conformation proposed for des-Trp[1],Nle[12]-minigastrin.

extend the α-helix throughout the molecule according to Chou–Fasman analysis. Instead, the α-helix is interrupted at Glu[6], leaving a flexible region in the center of the molecule with free rotation about the φ and ψ angles of Glu[6] and Ala[7]. The possibility of a flexible hinge in the central portion of little gastrin was also discussed by Torda et al. (1985), although at the Gly residue, not in the position that our data suggest. This flexibility might allow interaction between the two helical regions at either end of the molecule. An interaction of this sort may provide an explanation of the unusually high chemical shift of one of the Glu amides, Glu[3] or Glu[4]. A ring current shift due to the close proximity of an aromatic residue in the C-terminal portion may cause this unusual behavior. Furthermore, the very short α-helix segment at the N-terminus followed by a flexible region could not exist without some stabilizing interaction with the rest of the molecule. An interaction between the two terminal segments of the peptide chain might provide this stabilization.

The titration of des-Trp[1],Nle[12]-minigastrin from TFE to H_2O, followed by CD and NMR, shows two regions of conformational change. From 20% to 25% water, a cooperative conformational change is evident from the plot of the relative variation of the CD signal versus solvent composition (Figure 8). Accordingly, the slopes of the chemical shifts of the amide resonances plotted versus solvent composition (Figure 3) show an abrupt change at 20% H_2O. This conformational change involves the destruction of two hydrogen bonds; the absolute values of the temperature coefficients of Phe-NH and one of the Glu NH's are low at 10% H_2O and become high at 31% H_2O (Table I). The hydrogen bond involving Asp-NH is also weakened as indicated by an increase of its temperature coefficient from −1.8 to −3.0 ppb/K.

The second conformational change occurs from 60 to 100% H_2O. The relative molar ellipticity changes linearly in this region (Figure 8), indicating a noncooperative change. The CD spectra show a decrease of the intensity of the $n-\pi^*$ band and a blue shift in the position of the negative maximum. In 100% H_2O, the CD spectrum shows that the peptide structure is completely random. The chemical shifts of the amide protons versus solvent composition indicate a conformational change starting at approximately 70% H_2O in near accordance with the CD data. The slopes due to most of the amide resonances change sharply at this point (Figure 3). No hydrogen bonds remain in 100% H_2O.

From 30% water to about 60% water, the CD pattern of the gastrin analogue does not change, indicating that no conformational change is taking place (Figures 7 and 8). Consistent with these results, the positions of the amide resonances move slightly and in a linear fashion with the solvent composition in this region.

The determination of the conformation of des-Trp[1],Nle[12]-minigastrin in TFE may reveal the structural features needed for the full biological potency of the gastrin hormones. A conformational model has been proposed with two helical sections at the chain ends. Both helices might be stabilized by mutual interaction.

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Registry No. Des-Trp[1],Nle[12]-minigastrin, 75679-13-9.

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