Conformational Studies of Human [15-2-Aminohexanoic acid]little Gastrin in Sodium Dodecyl Sulfate Micelles by ^{1}H NMR†

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ABSTRACT: Human little gastrin is a 17 amino acid peptide that adopts a random conformation in water and an ordered structure in sodium dodecyl sulfate (SDS) micelles as well as in trifluoroethanol (TFE). The circular dichroism spectra in these two media have the same shape, indicative of a similar preferred conformation [Mammi, S., Mammi, N. J., Foffani, M. T., Peggion, E., Moroder, L., & Wünsch, E. (1987) *Biopolymers 26*, S1–S10]. We describe here the assignment of the proton NMR resonances and the conformational analysis of [Ahx^{15}]little gastrin in SDS micelles. Two-dimensional correlation techniques form the basis for the assignment. The conformational analysis utilizes NOE's, NH to C$^\alpha$H coupling constants, and the temperature coefficients of the amide chemical shifts. The NMR data indicate a helical structure in the N-terminal portion of the peptide. These results are compared with the conformation that we recently proposed for a minigastrin analogue (fragment 5–17 of [Ahx^{15}]little gastrin) in TFE.

Human little gastrin is a hormone that serves a variety of functions in the digestive process (Walsh & Grossman, 1975). It is a heptadecapeptide of the following sequence:

1 2 3 4 5 6-10 11 12 13 14 15 16 17

pGlu-Gly-Pro-Trp-Leu-[Glu]$_5$-Ala-Tyr-Gly-Trp-Met-Asp-Phe-NH$_2$

Although at reduced potency, the C-terminal tetrapeptide is sufficient to perform the wide range of activities of gastrins, while the role of the N-terminal portion is still not clear.

The conformation of little gastrin and related fragments has been extensively studied in water and TFE[1] by means of CD and ^{1}H NMR (Peggion et al., 1981, 1985; Mammi et al., 1988, 1989; Torda et al., 1985). While the conformation of this peptide in water is completely random (Peggion et al., 1981; Torda et al., 1985), the CD spectra in TFE indicate the presence of an α-helical segment (Peggion et al., 1981). Recently, we studied the conformational preferences of the C-terminal tridecapeptide in which the Met residue was replaced by Ahx (Mammi et al., 1988). This substitution does not affect the biological activity of the hormone (Morley et al., 1964) nor its conformation (Peggion et al., 1981) and prevents oxidation problems encountered with Met. We proposed that the N-terminal portion of des-Trp^1-[Ahx^{12}]-minigastrin adopts an α-helical structure, interrupted by the presence of Gly, with the C-terminal half of the molecule adopting a 3_{10}-helix. These two short helices might then be stabilized by mutual interaction. The elongation from minigastrin to little gastrin causes an extension of the α-helical segment at the N-terminus as indicated by CD.

The importance of membranes in the interaction of bioactive peptides with their receptors has been recognized in the past few years [Schwyzer (1986) and references cited therein]. The interaction with a cell membrane could cause the onset of a preferred conformation in a given peptide, thus facilitating recognition by its receptor. This hypothesis has led a number of researchers to study the secondary structure of such peptides in membranes and membrane-like environments such as surfactant micelles.

In previous work (Mammi et al., 1987), we studied the conformational properties of [Ahx^{15}]little gastrin in aqueous solutions containing SDS micelles by fluorescence and CD. From the blue shift of the maximum of Trp fluorescent emission, we showed that the hormone penetrates the micelles when the side chains are uncharged. The CD spectra of [Ahx^{15}]little gastrin in aqueous solutions in the presence of SDS micelles and in TFE have the same shape, but the intensity of the two negative maxima is 25% lower in the organic solvent (Mammi et al., 1987; Wu & Yang, 1978). In this paper, we report on the conformation of [Ahx^{15}]little gastrin in SDS micelles as studied by ^{1}H NMR.

Materials and Methods

The sample of [Ahx^{15}]little gastrin was provided by Prof. E. Wünsch of the Max-Planck-Institut für Biochemie, Munich (Moroder et al., 1983), and perdeuteriated SDS (SDS-d_{25}) was purchased from MSD Isotopes. *N*-Capryl-L-tryptophan ethyl ester was prepared by condensation of caprylic acid *N*-succinimidyl ester with L-tryptophan ethyl ester in dimethylformamide according to standard procedures (Wünsch, 1974).

Circular dichroism spectra were recorded on a Jasco J-600 spectropolarimeter; fluorescence measurements were carried out with a Perkin-Elmer Model MPF-66 fluorescence spectrophotometer. The NMR spectra were obtained on a Bruker AM 400 instrument equipped with an Aspect 3000 computer and operating at 400 MHz for protons. Processing was performed either on an Aspect 3000 or on a Bruker X-32 computer. Third-order polynomial baseplane correction was performed in the latter case. Peak positions were measured relative to TMS as internal standard. The NMR samples were prepared at peptide concentrations varying from 2.8 to 3.3 mM

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[1] Abbreviations: NMR, nuclear magnetic resonance; CD, circular dichroism; SDS, sodium dodecyl sulfate; TFE, trifluoroethanol; TMS, tetramethylsilane; COSY, two-dimensional homonuclear correlation spectroscopy; HPLC, high-performance liquid chromatography; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; FID, free induction decay; TOCSY, total correlation spectroscopy; cmc, critical micellar concentration; Ahx, 2-aminohexanoic acid; ppb, parts per billion.

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in 0.3 M SDS-d_{25}, either in 90% H_2O–10% D_2O at pH ≈3.0 or in 100% D_2O at pH ≤2.0. After several weeks from the preparation of the solutions, minor peaks appeared in the NMR spectra, and HPLC analysis showed decomposition of the sample. Only fresh solutions were employed for NOESY spectra.

Phase-sensitive COSY experiments were performed in H_2O according to Marion and Wüthrich (1983). The data were collected as 320 FID's of 352 scans each with irradiation of the water peak throughout the entire sequence except during acquisition. Prior to transformation, the 2K × 320 data matrix was zero filled to 16K in t_2 and to 2K in t_1; a sine bell window function was applied in t_2, and a shifted sine bell function ($\pi/8$) was used in t_1.

The 2D homonuclear Hartmann–Hahn experiment (TOCSY) in H_2O was performed with the sequence described by Bax and Davis (1985). The MLEV-17 spin-locking sequence was cycled 14 times, and the "trim" pulses were 3 ms each for a total mixing time of 75 ms. The spin-locking power was $\gamma B_2/2\pi \approx 3.33$ kHz. A final spectrum of 1K by 1K real data points was obtained from 448 FID's of 128 scans each and zero filling in the t_1 dimension. Gaussian multiplication was applied in both dimensions prior to transformation. For the D_2O sample, the same experiment was performed with a total mixing time of 77 ms with a spin-locking power of $\gamma B_2/2\pi \approx 3.23$ kHz. For each of the 512 FID's, 176 scans were collected.

A relayed COSY experiment was performed in H_2O according to the sequence of Eich et al. (1982), a delay of 30 ms being used and 256 FID's of 416 scans each being collected. Zero filling was performed in the t_1 dimension to give a final real matrix of 1K by 1K points. Several phase-sensitive NOESY experiments (Jeener et al., 1979; Bodenhausen et al., 1984) were collected at mixing times of 80, 120, 180, 200, and 250 ms (Kumar et al., 1981). The notation for proton–proton distances is according to Wüthrich et al. (1984).

All experiments were performed at 49 °C where the best resolution between the various NH's was achieved and all the peaks were sufficiently sharp. Temperature-dependence studies were carried out between 25 and 65 °C. In all experiments in H_2O, the solvent signal was suppressed by presaturation.

RESULTS

CD and Fluorescence Measurements

The CD spectrum of every NMR sample was identical with those at a peptide concentration of ≈0.1 mM, indicating that aggregation did not take place at the concentration used for NMR. In addition, at room temperature and at 49 °C, [Ahx[15]]little gastrin displays identical CD spectra. This fact strongly indicates that the same conformer population is present at both temperatures.

As reported previously (Mammi et al., 1987), solubilization of [Ahx[15]]little gastrin in the interior of detergent micelles was monitored by the blue shift from 356 to 340 nm of the Trp fluorescence maximum. Also capryl-L-Trp-OEt in the presence of SDS above the cmc has an emission maximum at 340 nm, demonstrating that it penetrates the detergent micelles.

^1H NMR Measurements

Spin System Identification. A combination of three experiments performed on a 90% H_2O solution is shown in Figure 1: a phase-sensitive COSY, a TOCSY, and a relayed COSY. From the TOCSY experiment, all the spin systems of the aromatic amino acids and those of Ala and Asp were identified, but no conclusive cross peak was found to identify the spin systems of the Glu's or those of Leu and Ahx. However, the complete assignment was accomplished with the relayed COSY. The sequence-specific assignment of the Glu residues and that of the AMXX′ spin systems were performed with the aid of NOESY experiments (discussed below).

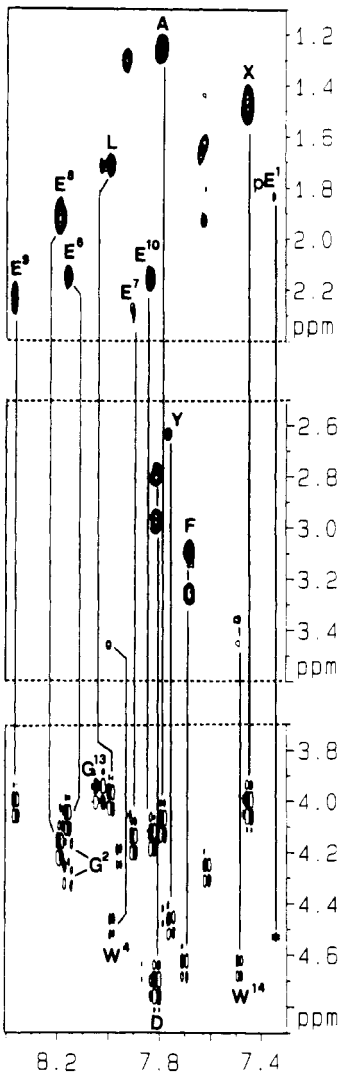

FIGURE 1: Portions of a phase-sensitive COSY (bottom), TOCSY (middle), and relayed COSY (top) with the assignment of the spin systems. The spectra were run on a 3.19 mM solution of [Ahx[15]]little gastrin in 319 mM SDS-d_{25} in 90% H_2O at 49 °C. A mixing time of 75 ms was used for the TOCSY, and a delay of 30 ms was used for the relayed COSY. Abbreviations follow the standard one-letter code, and X stands for 2-aminohexanoic acid.

The $C^{\alpha}H$ to aliphatic side chain region of a TOCSY experiment with 77-ms mixing time performed on a D_2O solution is shown in Figure 2. From this spectrum, all the resonances of the protons in the side chains were assigned.

NOESY Experiments. The observed nuclear Overhauser effects were used qualitatively to perform the sequential assignment of residues and derive conformational information. In Figure 3, the NH to C^{α} proton region of a NOESY experiment (80-ms mixing time) is shown. Comparison with Figure 1 allows the complete sequential assignment as illustrated in the figure. Strong $d_{\alpha N}$ connectivities were found for i = 1, 3, and 12–16. In contrast, weak or very weak $d_{\alpha N}$ peaks were identified for residues 5–11.

Figure 4 shows the NH and aromatic region of a NOESY experiment (200-ms mixing time). Cross peaks connect sequentially all of the NH's from Leu[5] to Glu[10] and from Tyr[12] to Trp[14]. The assignments described above are therefore confirmed. The majority of the peaks were also present in

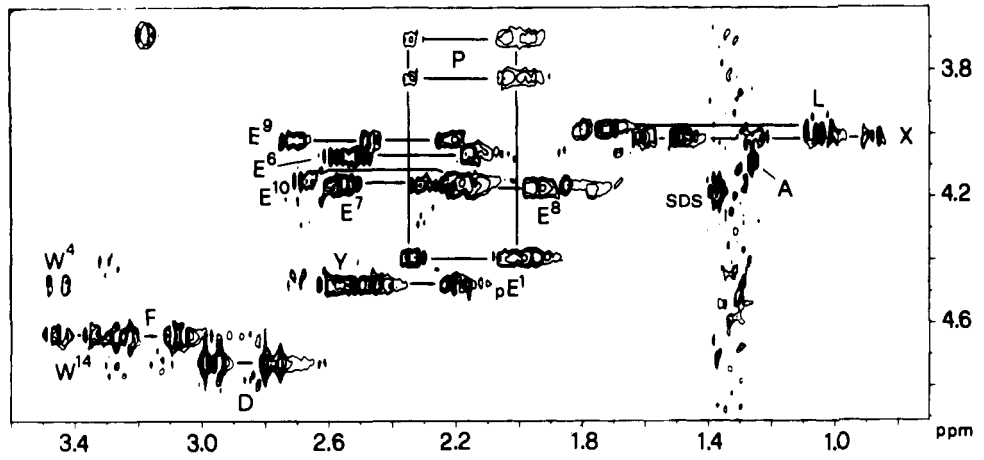

FIGURE 2: Aliphatic region of a TOCSY spectrum (77-ms mixing time) of a 2.82 mM solution of [Ahx[15]]little gastrin in 326 mM SDS-d_{25} in D_2O at 49 °C. Abbreviations follow the standard one-letter code, and X stands for 2-aminohexanoic acid.

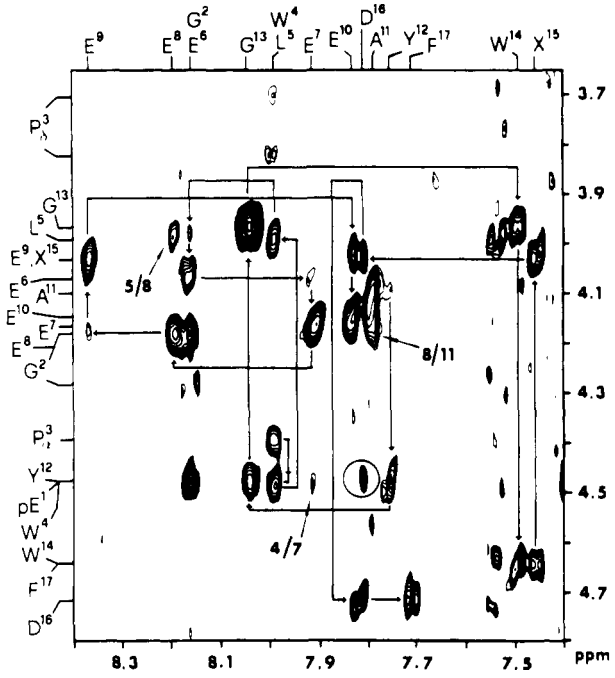

FIGURE 3: Fingerprint region of a pure-phase absorption NOESY spectrum (80-ms mixing time) of a 3.29 mM solution of [Ahx[15]]little gastrin in 328 mM SDS-d_{25} in 90% H_2O at 49 °C. The sequential NH to α assignment from Pro[3] to Phe[17] is illustrated. Arrows point at $d_{\alpha N}(i,i+3)$ connectivities, and the long-range cross peak involving Asp[16] NH is circled (see text). Abbreviations follow the standard one-letter code, and X stands for 2-aminohexanoic acid.

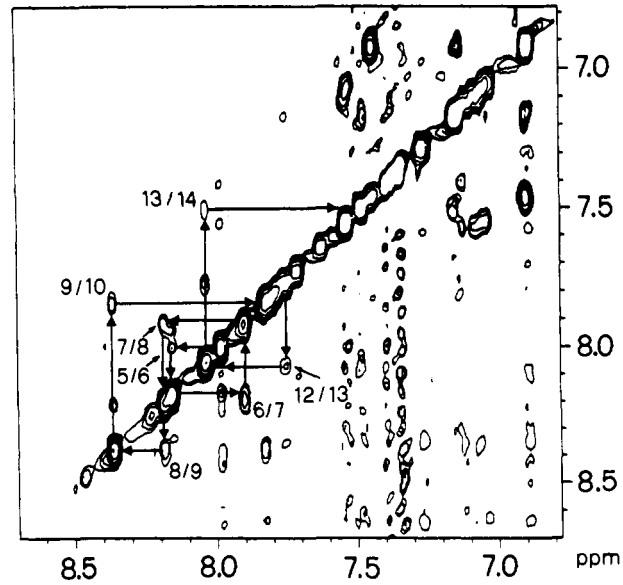

FIGURE 4: Amide and aromatic region of a pure-phase absorption NOESY spectrum (200-ms mixing time) of a 3.19 mM solution of [Ahx[15]]little gastrin in 319 mM SDS-d_{25} in 90% H_2O at 49 °C. The NH to NH connectivities are illustrated by their position in the sequence.

spectra at shorter mixing times with reduced intensities. This indicates that spin diffusion is not present under these conditions.

The two C-terminal NH protons are obscured by the resonances of the aromatic side chains at 49 °C (δ_{trans} = 7.43 ppm; δ_{cis} = 6.90 ppm). The *cis*-NH was identified during the temperature study; a NOESY cross peak allowed the identification of the *trans*-NH. This peak cannot be assigned to aromatic protons because it was not found in an analogous experiment in D_2O. No cross peak indicates that any two aromatic rings are close in space.

Medium-range NOE connectivities were identified in experiments with mixing times varying from 80 to 250 ms. Even at the shortest mixing time utilized, interactions of the type $d_{\alpha N}(i,i+3)$ ranging from Trp[4] to Ala[11] were found. Severe overlap in the aliphatic region prevented the unambiguous detection of $d_{\alpha\beta}(i,i+3)$ cross peaks. The relevant NOESY connectivities are highlighted in Figure 3 and reported in Table I.

Table I: Summary of Temperature Coefficients, Coupling Constants, and NOE Connectivities Determined for Human [Ahx[15]]little Gastrin in SDS Micelles

	pE	G	P	W	L	E	E	E	E	E	A	Y	G	W	X	D	F
$-\Delta\delta$(NH)/ΔT[a]	–	8.6	–	8.6[b]	8.6[b]	9.7	2.1	5.4	8.0	2.1[c]	2.6[c]	4.4	5.7	4.2	–	3.2[c]	3.4
	–	(0.2)	–	(0.5)	(0.5)	(0.7)	(0.3)	(0.5)	(0.4)	(0.8)	(0.6)	(0.3)	(0.4)	(0.1)	–	(0.4)	(0.1)
$^3J_{NH\text{-}C^\alpha H}$[d]	–	–	–	5.3	5.3	5.1	5.4	5.6	5.2	5.7	5.5	7.5	–	6.1	6.4	7.3	6.9
$C^\alpha H(i)$-$NH(i+1)$	|—|		|—|										|—|—|—|—|—|		
$NH(i)$-$NH(i+1)$[e]			|—|	|—|—|—|—|—|							|—|—|			
$C^\alpha H(i)$-$NH(i+3)$			|———|														
				|———|													
									|———|							

[a] Temperature coefficients in ppb/K. Errors in parentheses represent 95% confidence intervals. [b] Overlapping resonances. [c] Can be interchanged. [d] Coupling constants are in Hz ± 1 Hz. [e] For proline, the δ-protons are used instead of the NH.

Only one long-range NOE was identified, apparently connecting Asp[16] NH and the $C^\alpha H$ of either Tyr[12], Trp[4], or pGlu[1]. Its singlet appearance makes it origin uncertain, and overlap in the $C^\alpha H$ region prevents specific assignment.

Temperature-Dependence Study. Increasing the temperature from 25 to 65 °C caused a sharpening of all the resonances of both the peptide and the solvent. This is indicative of increased mobility due to the reduced viscosity of the solution. In fact, CD results show that no conformational changes take place with increased temperature (see above).

The temperature coefficients of amide protons are reported in Table I. The dependence on temperature is linear in all cases. Temperature coefficients could not be determined for Trp[14], Ahx[15], and the trans C-terminal amide because of overlapping aromatic resonances. The largest temperature dependence was observed for Gly[2], Trp[4], Leu[5], and Glu[9] NH's. The value for the cis C-terminal amide (not reported in the figure) is 6.1 ± 0.2 ppb/K. All other amide resonances have temperature coefficients in the range −2.1 to −5.7 ppb/K. The temperature coefficient of the amide proton of the model compound capryl-L-Trp-OEt (4.18 mM in 300 mM SDS) was −7.9 ± 0.4 ppb/K.

NH to $C^{\alpha}H$ Coupling Constants. The $^3J_{NH-C^{\alpha}H}$ were determined at 49 °C from the phase-sensitive COSY spectrum in the following way: each cross section (row) containing a cross peak in the upper-left quadrant was deconvoluted with Lorentian functions. The separation in the two calculated antiphase components was then averaged for all the rows that contained any given cross peak, and the average values are reported in Table I. The coupling constants of the residues from Trp[4] to Ala[11] are lower than 6.0 Hz. The highest values are observed for Tyr[12], Asp[16], and Phe[17].

Discussion

The determination of the biologically important conformation of small, linear peptides is often more difficult than that of proteins. It is difficult to find suitable solvent systems that lend themselves to spectroscopic study and at the same time impart on the peptide a specific structure that is relevant to its biological activity. In addition, the flexibility of small peptides permits a number of interchanging conformers. The structural information derived from spectroscopic techniques therefore reflects an average of conformations, and many diagnostic features of ordered conformations can be obscured by the presence of disordered structures and by local fluctuations around the ordered ones. Several techniques need to be employed for these types of studies, and evidence for ordered conformation should not be sought by one method alone.

In the case of the gastrins, we have shown that water is not a structure-supporting environment (Peggion et al., 1981, 1985). We have also shown by CD that TFE is able to induce an ordered conformation on gastrin peptides from 8 to 17 amino acid residues long and that the increase in structural order with chain length parallels the increase in biological activity (Peggion et al., 1985). The CD spectra of little gastrin are compatible with the presence of approximatively 30% α-helix although it is not possible to determine whether this is an ensemble average or reflects partial ordering of each molecule. The observation that TFE and SDS micelles are able to support a similar conformation (Mammi et al., 1987; Wu & Yang, 1978) as shown by CD suggests that this conformation could be adopted by these peptide hormones in the presence of natural membranes in the proximity of their receptors. This conformation could favor the recognition and therefore enhance the activity of the hormones.

A difficulty in the use of SDS micelles in water as a solvent system for NMR studies is the broadening of all resonances, especially the water line, because of restricted molecular motion. We observe a doubling of the line width of the water resonance even when very good field homogeneity is obtained. This makes water suppression a more serious problem, requiring longer preirradiation times at lower power and causing severe baseplane distortions in two-dimensional experiments.

A concurring problem is the viscosity of these solutions due to the high concentration required for NMR measurements. We used solutions containing 3 mM peptide, which required the presence of 3 mM micelles, or about 0.3 M SDS, to ensure complete solubilization of the hormone into the micelles without the risk of self-aggregation of the peptide.

These problems were partially alleviated by working at high temperature where the lower viscosity of the sample caused a reduction of line widths and the increased signal to noise ratio allowed the acquisition of two-dimensional NMR experiments in a reasonable amount of time.

The classic NOESY cross peaks utilized for the determination of α-helical conformation are those corresponding to $d_{\alpha N}(i,i+3)$ and $d_{\alpha\beta}(i,i+3)$. Overlap of aliphatic resonances associated with the redundancy of residues present in the sequence allowed the unambiguous identification of only a few of the $d_{\alpha N}(i,i+3)$ cross peaks. Specifically, short distances of the type $d_{\alpha N}(i,i+3)$ were found for $i = 5$ and 8. A very weak cross peak of this type was also found for $i = 4$. Unfortunately, none of the $d_{\alpha\beta}(i,i+3)$ cross peaks were identified unambiguously. The detailed determination of the location of the helical stretch has to rely therefore on different information.

Many results indicate that residues from Trp[4] to Ala[11] adopt a helical conformation. In addition to the cross peaks mentioned above, the $^3J_{NH-C^{\alpha}H}$ coupling constants of all these residues are <6.0 Hz, and d_{NN} connectivities are present together with very weak $d_{\alpha N}$ peaks.

The observation of small coupling constants and d_{NN} cross peaks for a single amino acid are not diagnostic for α-helical conformation. However, if several sequential residues meet these criteria, the uniqueness of identification increases substantially (Wüthrich et al., 1984; Pardi et al., 1984). Moreover, in random conformations, $d_{\alpha N}$ and d_{NN} cross peaks have similar intensities (Wüthrich et al., 1984), while in this study the former are much weaker.

The secondary structure of the residues from Gly[13] to Phe[17] is less defined. We found no medium-range NOE's while strong $d_{\alpha N}$ cross peaks are present. The coupling constants also do not provide evidence for a specific conformation. The last four residues seem to adopt mainly a random conformation, as indicated by strong $d_{\alpha N}$ cross peaks. All the data on the tetrapeptide -Ala-Tyr-Gly-Trp- are consistent with the type III β-turn previously proposed although its presence cannot be proven unambiguously. Specifically, the value of the coupling constant is high for the Tyr residue (7.5 Hz) and lower for Ala and Trp[14] (5.5 and 6.1 Hz, respectively). Moreover, d_{NN} cross peaks are present for $i = 12$ and 13, but no medium-range NOE's were found, such as those corresponding to $d_{NN}(i,i+2)$ or $d_{\alpha N}(i,i+3)$.

The long-range NOE that involves Asp NH is potentially of interest, but its coupling pattern and overlap in the $C^{\alpha}H$ region prevent definitive conclusions. A cross peak connecting Asp NH and Tyr $C^{\alpha}H$ would indicate the presence of secondary structure in the C-terminal portion of the molecule. On the other hand, a proximity between Asp NH and Trp[4] or pGlu[1] $C^{\alpha}H$ would indicate an interaction between the two ends of the molecule as proposed previously (Mammi et al., 1988, 1989).

It is generally accepted that high values of the temperature coefficients of the amide protons reflect exposure to the solvent, while low values indicate shielding, because of intramolecular hydrogen bonding or burial in hydrophobic pockets (Kopple

et al., 1969; Ohnishi & Urry, 1969). In the micellar system, the situation is more complicated since it is not apparent whether an amide proton which is not involved in an intramolecular hydrogen bond is solvated by water or surrounded by the hydrophobic SDS molecules. In an effort to clarify this point, we determined the temperature coefficient of the amide proton of capryl-Trp-OEt. This molecule was chosen because its hydrophobic tail allows it to penetrate the micelles and it is possible to monitor this process by fluorescence. The only amide proton present in this molecule should not be involved in an intramolecular hydrogen bond and is therefore a good model for solvent-exposed amides. The high value that we found (−7.9 ppb/K) seems to indicate that solvent-exposed amide protons behave similarly in water and micelles. Unfortunately, no temperature coefficient has been reported in the literature for compounds that are certainly involved in intramolecular hydrogen bonds in the presence of micelles. Therefore, the value of −3.0 ppb/K, normally accepted as a cutoff between solvent-exposed and solvent-shielded amide protons in water, may not be applicable in the micellar system.

For little gastrin, we conclude that the amide protons of Gly^2, Trp^4, Leu, Glu^6, and Glu^9 are solvent exposed. The lower coefficients of many of the other protons in the N-terminal region agree with the presence of a helical segment from residue 4 to residue 11. Local fluctuations and the fact that amide protons in this conformation are not completely hindered from the solvent can account for the higher value of some coefficients.

The tendency of the N-terminal portion to fold into a helical conformation in a hydrophobic environment might be a necessary element for the transport of the molecule into the membrane. This may facilitate the recognition by the receptor and therefore be responsible for the full activity of the hormone.

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