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The structure of the rodent and porcine neuropeptide galanin and antagonists as determined by FTIR and CD spectroscopy[1,2]

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FT-IR spectroscopy was used to study the conformation of the porcine neuropeptide galanin, fragments 1–16 of the porcine and human peptides and the antagonists M15 and M35. All peptides were shown to be structureless in aqueous solution. Upon addition to SDS micelles, only porcine galanin and the fragment consisting of amino acids 1–16 showed any evidence of interaction, adopting a helical structure. No interaction could be demonstrated with zwitterionic lipids for any peptide except M15 which formed a thermally unstable helical conformation which unfolded promoting aggregation at around 45°C.

Additional studies on rat galanin in various solvent systems were made by using circular dichroism spectroscopy. The results obtained support the observations made by FT-IR spectroscopy.

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On a utilisé la spectroscopie IR par FT pour étudier la conformation du neuropeptide de porc, galanine, des fragments 1–16 des peptides humain et de proc et les antagonistes M15 et M35. On a montré que, en solutions aqueuses, tous les peptides sont sans structure. Par addition de micelles SDS, seule la galanine de porc et le fragment comportant les acides aminés 1–16 ont présenté des signes d'interaction en adoptant une structure hélicoïdale. Aucun des lipides zwitterioniques, à l'exception du M15 que donne une conformation hélicoïdale thermiquement instable et qui se défait avec une promotion de l'agrégation autour de 45°C, ne démontre d'interaction.

Faisant appel au dichroïsme circulaire, on a fait des études supplémentaires sur la galanine de rat dans divers systèmes. Les résultats obtenus confirment les observations faible à l'aide de l'IR par FT.

[Traduit par la rédaction]

Introduction

Galanin is a 29 amino acid neuropeptide initially isolated from the small intestine of the pig (1) and since located in various parts of the central and peripheral nervous system (2), including neurons in the respiratory, gastrointestinal and urogenital tracts, adrenal medulla and pancreas. The amino acid sequences of rat, pig, and human galanin (1) have been determined (Table 1). The first 15 residues are absolutely conserved in all species and receptor affinity has been correlated with this sequence. In particular, tryptophan in position 2 is crucial for receptor binding (3). All galanins, with the exception of the human isoform, are secreted as C-terminal amides. As the sequences bear no homology to any other family of neuropeptides, it appears that galanin is the first member of a new family of neuropeptides.

The physiological effects of galanin are diverse and vary with species. Depending upon species, galanin may modulate the physiological effects of growth hormone, insulin, glucagon, prolactin, somatostatin, and dopamine (4). Evidence exists that galanin functions as a neurotransmitter in the pancreas in some animals, with a possible role in the neural regulation of islet function as part of the metabolic response to stress (5). There are indications that the inhibitory effect of galanin on insulin secretion results from an action of the peptide on K^+ channels and indirectly, on Ca^{2+} channels within the β-cells (6). In addition, galanin is an effective inhibitor of substance P. Biochemical characterization of the galanin receptor shows that it is a glycoprotein of 54 kDa belonging to the G-protein coupled class of receptors (7). The involvement of a G-protein in galanin-mediated insulin suppression has been suggested (8).

[1]NRCC No. 34717.

[2]Abbreviations used: FT-IR, Fourier transform infrared; CD, circular dichroism; DHPC, dihexadecylphosphatidylcholine; DOPC, 1,2-dioleoyl-sn-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol; HFP, 1,1,1,3,3,3-hexafluoro-2-propanol; TFE, trifluoroethanol; SDS, sodium dodecyl sulphate.

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TABLE 1. Amino acid sequences of porcine, rat, and human galanin and of the antagonists M15 and M35

	1				5					10					15
Porcine	Gly	Trp	Thr	Leu	Asn	Ser	Ala	Gly	Tyr	Leu	Leu	Gly	Pro	His	Ala
Human	Gly	Trp	Thr	Leu	Asn	Ser	Ala	Gly	Tyr	Leu	Leu	Gly	Pro	His	Ala
Rodent	Gly	Trp	Thr	Leu	Asn	Ser	Ala	Gly	Tyr	Leu	Leu	Gly	Pro	His	Ala
M15	Gly	Trp	Thr	Leu	Asn	Ser	Ala	Gly	Tyr	Leu	Leu	Gly	Pro	**Gln**	**Gln**
M35	Gly	Trp	Thr	Leu	Asn	Ser	Ala	Gly	Tyr	Leu	Leu	Gly	Pro	**Pro**	**Pro**

	16				20					25					30	
Porcine	Ile	Asp	Asn	His	Arg	Ser	Phe	His	Asp	Lys	Tyr	Gly	Leu	Ala		NH_2
Human	**Val**	**Gly**	Asn	His	Arg	Ser	Phe	**Ser**	Asp	Lys	**Asn**	Gly	Leu	**Thr**	**Ser**	COOH
Rodent	Ile	Asp	Asn	His	Arg	Ser	Phe	**Ser**	Asp	Lys	**His**	Gly	Leu	**Thr**		NH_2
M15	**Phe**	**Phe**	**Gly**	**Leu**	**Met**											NH_2
M35	**Gly**	**Phe**	**Ser**	**Pro**	**Phe**	**Arg**										NH_2

The widespread physiological effects of galanin have stimulated interest in the preparation of pharmacologically useful derivatives, particularly given its possible use in pain modulation due to its antagonism of substance P (3). Such an undertaking will be greatly facilitated by an understanding of the relationship between galanin structure and function. Infrared and circular dichroism spectroscopy are valuable tools for obtaining semi-quantitative information concerning the secondary structure of proteins and peptides in solution. We have used these techniques to assess the conformation of rat and porcine galanin, the first 16 amino acids of porcine and human galanin as C-terminal acids as well as that of analogues composed of the first 13 amino acids of galanin and either the final 7 amino acids from the substance P sequence (M15) or the final 8 amino acids from the bradykinin sequence (M35) as C-terminal amides in aqueous solution. The amphipathic nature of galanin suggests the possibility of interaction with membranes. In addition, we have therefore studied the interaction of galanin with model membrane systems to assess the nature of any conformational changes induced upon interaction with membranes.

Experimental

Materials

Galanin and its analogues (see Table 1) were synthesized by solid phase synthesis or purchased from Sigma (St. Louis, MO). The purity of synthetic peptides was determined by HPLC chromatography and the amino acid sequence confirmed. Sodium dodecyl sulfate (SDS) was purchased from BDH Ltd (Poole, England), 2,2,2-Trifluoroethanol (TFE) from E. Merck (Darmstadt, Germany), and 1,1,1,3,3,3-hexafluoro-2-propanol (HFP) from Sigma. The lipids 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-sn-glycero-3-phosphoglycerol (DOPG) were obtained either from Sigma or Avanti Polar Lipids (Pelham, AL).

Methods

FT-IR studies. Peptide samples in aqueous solution were prepared by dissolution of the peptide in TRIS buffered saline (50 mM TRIS, 150 mM NaCl, pD 7.6: TBS) to a final concentration of 10 mg/mL. Liposomes were prepared by sonication of lipids in the appropriate buffer and liposome suspensions added to the required amount of peptide to yield a lipid-to-protein ratio of 50:1 and a final peptide concentration of 10 mg/mL. Spectra were recorded on a Digilab FTS-60 Fourier transform infrared spectrometer equipped with a liquid-nitrogen cooled mercury–cadmium–telluride detector and continuously purged with dry nitrogen. Approximately 3 μL of sample was placed between a pair of CaF_2 windows separated by a 50 μm Mylar spacer and mounted in a Harrick demountable cell. Temperature was maintained by a circulating water bath. For each sample 100 interferograms were recorded, co-added, and Fourier transformed to generate a spectrum of resolution 2 cm^{-1}. Buffer spectra were recorded under identical conditions and interactively subtracted from sample spectra. Residual water vapour was interactively subtracted. All spectra were subjected to Fourier self-deconvolution performed as described previously (9) using a half width of 13.5 cm^{-1} and a resolution enhancement factor $k = 1.7$.

CD-studies. Rat galanin solutions with a concentration of approximately 25 μM were titrated with SDS and phospholipid vesicle solutions prepared from DOPC and DOPG. In the titration with SDS a 10 mM phosphate buffer (pH = 7.0) was used, in all other cases water was used. CD-spectra of the peptide were recorded in solvents with 0, 1, 2, 5, 10, and 20 mM SDS and with 0, 0.025, 0.05, 0.1, 0.2, 0.5, 1.0, and 2.0 mg/mL of vesicle solution. CD-spectra of the peptide in 100% TFE and 100% HFP were also recorded. CD-spectra were recorded using a Jasco-720 spectropolarimeter with 2 mm Hellma quartz cuvettes at a temperature of 25°C. A resolution of 0.5 nm and a scan speed of 10 nm per min were used. The acquired spectra were the average of three continuous scans, usually measured between 185 and 250 nm, and corrected with respect to baseline and for dilution. Quantitative evaluation of CD spectra is dependent upon a knowledge of the extinction coefficient of the peptide. This parameter was determined for galanin, dissolved in water, by quantitation of the peptide using amino acid analysis and light absorption spectroscopy and yielded a value of $\epsilon_{278} = 6620\ M^{-1}\ cm^{-1}$.

Results and discussion

The FT-IR spectrum of porcine galanin in TBS buffer (pD 7.6) is shown in Fig. 1. The amide I mode, predominantly (80%) a C=O stretching vibration, gives rise to a broad band centered at 1646 cm^{-1}. Such a position is characteristic of proteins and peptides which have little or no repetitive secondary structure, generally referred to as a "random" conformation (assignments

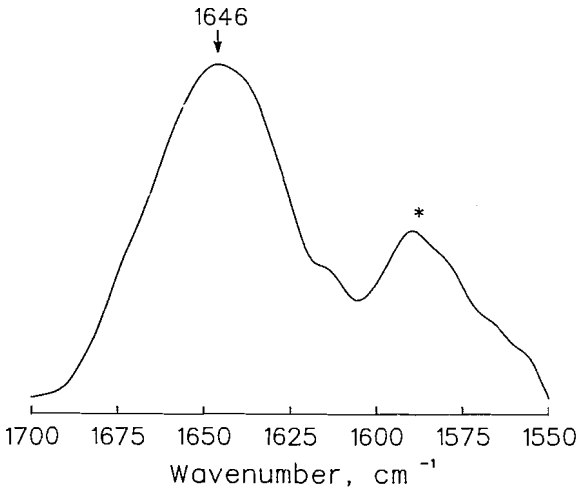

FIG. 1. Infrared spectrum of porcine galanin in TBS buffer, pD 7.6 in the spectral region 1550–1700 cm^{-1}.

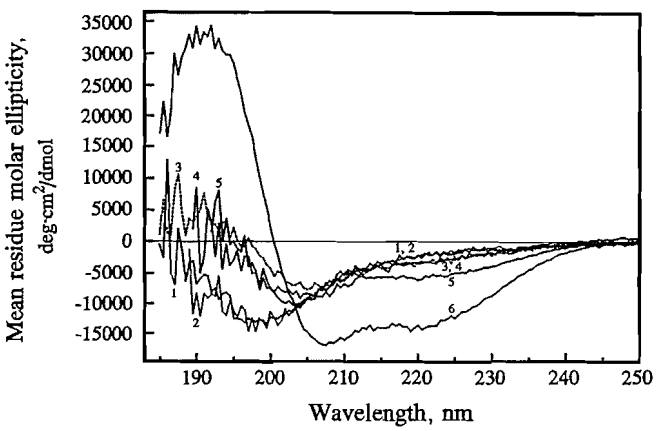

FIG. 2. Circular dichroism spectra of rat galanin at 25°C in pure water (*1*), in water containing 0.1 mg/mL DOPC (*2*), 10 mM SDS and 10 mM phosphate (*3*), 0.1 mg/mL DOPG (*4*), and in 100% HFP (*5*), or 100% TFE (*6*).

in this work are based on refs. 10–12). Useful structural information can also be obtained from the amide II mode, predominantly attributed to N—H bending vibrations, which gives rise to absorptions in the frequency range 1520 to 1560 cm^{-1}. The amide II mode is highly sensitive to the strength of hydrogen bonds involving the amide N—H groups, strong hydrogen bonds restricting the bending vibration and increasing the amide II frequency. In addition, upon dissolution in ^2H_2O, exchange of amide protons for solvent deuterons shifts the amide II band lower in frequency by approximately 100 cm^{-1}. The disappearance rate of the amide II band can therefore be a useful indicator of the degree of solvent exposure and the strength of any hydrogen bonds present, a high degree of solvent exposure and weak hydrogen bonds facilitating exchange. When galanin is dissolved in deuterated buffer the amide II band completely disappears, suggesting little if any hydrogen bonding involving the amide groups and a high degree of solvent exposure. Thus, analysis of both the amide I and the amide II bands suggests that galanin is unordered in aqueous solution. Circular dichroism spectra (Fig. 2) of rat galanin in aqueous solution are also indicative of a peptide with little or no long range order. The apparent lack of long range order in galanin is not surprising considering the high number of positive charges

TABLE 2. Percentages of α-helical structure of rat galanin as evaluated from CD spectra in pure water, in water containing 0.1 mg/mL DOPC, 0.1 mg/mL DOPG or 10 mM SDS as well as in pure hexafuropropanol and pure trifluoroethanol

Medium	% α-helix
H_2O	12
DOPC	13
DOPG	17
SDS	16
HFP	22
TFE	41

carried by the peptide, charge repulsion preventing folding into a more compact and energetically favorable conformation.

The spectral features in the frequency range 1560–1620 cm^{-1} arise from amino acid side chain vibrations (tyrosine, tryptophan, arginine, and aspartate). The absorption at 1590 cm^{-1} from arginine (marked with an asterisk) is particularly prominent.

Similar results were obtained from fragments 1–16 of human and porcine galanin as well as from the two analogues M15 and M35 (spectra not shown).

While the structure of a biological peptide in solution is important, it does not necessarily reflect the active conformation of the peptide. It is possible that unstructured peptides must in fact be induced to adopt an active, folded conformation. This may be achieved by structural rearrangements upon interaction with a recognition site. This recognition site may be part of an integral membrane protein (receptor) or the lipid bilayer of the cell membrane. To assess any conformational changes which may occur in the presence of membranes we recorded CD and IR spectra in a range of membrane mimetic environments.

Organic solvents are often used as membrane mimetic agents, usually to avoid the problems inherent in studying membrane suspensions with such techniques as NMR spectroscopy. The most commonly used organic solvent for such purposes is trifluoroethanol. It has been suggested that TFE provides a "helix-stabilizing" medium, stabilizing helical structures in polypeptide strands which have helix-forming propensity (13).

It has been shown by NMR spectroscopy (14) that rat galanin adopts a helical structure in TFE solution but not in aqueous solution. In agreement with this finding CD spectra (Fig. 2) of rat galanin in TFE are indicative of the presence of significant proportions of α-helical secondary structure. If we assume that the peptide chain contains only unordered and α-helical secondary structures (there is no evidence from CD or NMR spectra to suggest the presence of β-sheet structures) the amplitude at 222 nm can be used to determine the relative fraction of either (15, 16). From the spectra in Fig. 2 the percentages of α-helical structure in a variety of environments could be calculated (see Table 2). The highest proportion of helical secondary structure was found in TFE, followed by hexafluoropropanol. Only minor differences in ellipticity at 222 nm are seen between spectra in pure H_2O and those measured in the presence of SDS, DOPG, and DOPC.

The FT-IR spectrum of porcine galanin in TFE is shown in Fig. 3. Following deconvolution the amide I maximum is observed at 1658 cm^{-1}, a position indicative of the adoption of helical structure. The other analogues of galanin studied here

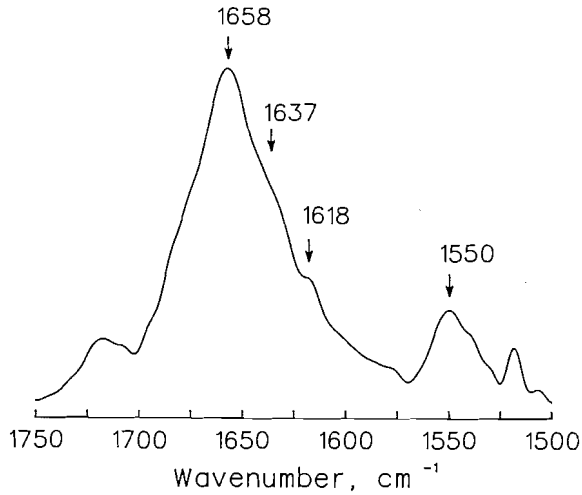

FIG. 3. Infrared spectrum of porcine galanin dissolved in TFE in the 1500–1750 cm^{-1} region.

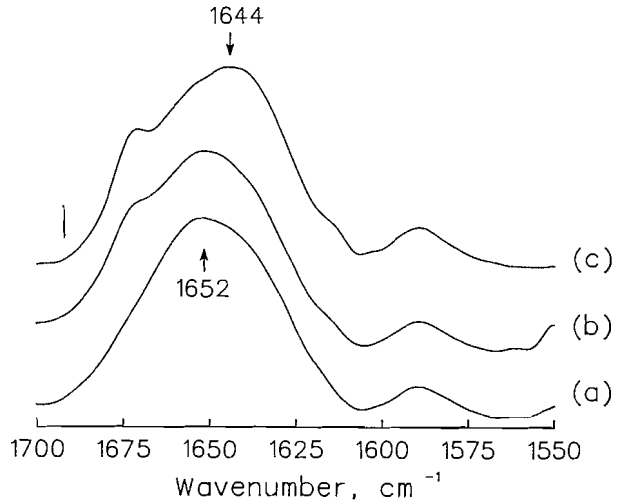

FIG. 4. Comparison of the infrared spectrum of porcine galanin (*a*) with porcine galanin fragment 1-16 (*b*) and human galanin fragment 1-16 (*c*), all in TBS buffer, pD 7.6 containing 5% SDS.

also adopted helical structures in TFE solution (spectra not shown). The frequency and small halfwidth of the amide I band suggest that helical structures are present in significant proportions (>75%). In addition, the position of the amide II band at 1550 cm^{-1} is also suggestive of significant helical structure. This result conflicts with the quantitative determination of the percentage of helices in galanin in TFE obtained from the CD data. This discrepancy may be related to the fact that in the CD calculations extinction coefficients obtained for helical and unordered polypeptide chains in aqueous solution were used for data obtained in organic solvents.

The presence of significant proportions of helical structures in peptides in TFE is often taken to indicate that the peptide is capable of adopting a helical secondary structure within the hydrophobic core of lipid membranes, i.e., is a reliable measure of its propensity to form helical secondary structures. However, we recently undertook a FT-IR spectroscopic examination of the effects of halogenated alcohols on the secondary structure of a variety of proteins and polypeptides (17). Each protein studied, whether it be predominantly helical, β-sheet or structureless in aqueous solution exhibited an amide I maximum at 1657 cm^{-1}. The adoption of helical secondary structures by peptide chains in TFE solution appears therefore to be the result of properties of TFE rather than any intrinsic properties of the polypeptide chain. Results obtained from studies of peptide conformation in halogenated alcohols should be treated with caution.

The weak absorption at 1618 cm^{-1} arises from tyrosine side chain vibrations. The additional feature in Fig. 3, at 1637 cm^{-1}, may have four possible origins. Firstly, it may be attributed to β-sheet structures. This is, however, unlikely, as preliminary NMR spectroscopic data show no resonances attributable to β-sheet in TFE solution (18). Secondly, a weak feature at 1637 cm^{-1} may be attributed to bound water present in the hygroscopic TFE (unpublished observations, M.J. H.M.). Thirdly, this feature may reflect hydrogen bonding of TFE to amide C=O groups. Finally, it may reflect the presence of β-turns. At present it is not possible to distinguish between the last three possibilities.

Another feature is apparent in spectra of galanin at around 1720 cm^{-1} in TFE solution (Fig. 3). This may be attributed to absorptions arising from the protonated COOH groups of aspartic acid residues present in galanin. The appearance of infrared bands arising from the protonated form of carboxylic acid side chains is a feature which we have consistently noted in spectra of proteins in TFE solution, which can be related to the acidic nature of this solvent. The relatively low frequency of the carbonyl absorption suggests that they are involved in hydrogen bonds, presumably with TFE. Protonation of and hydrogen bonding to side chains by TFE may be expected to have important structural consequences. Again, this illustrates the care with which data obtained for peptides in TFE solution should be treated.

More realistic attempts to study the interactions of peptides with membranes require the use of liposome or micellar systems. As galanin carries a net positive charge, any interaction would be expected to be specific for negatively charged liposomes or micelles. The spectrum of porcine galanin in the presence of 5% SDS is shown in Fig. 4*a*. The amide I maximum under these conditions is seen at 1652 cm^{-1}. Such an absorption in deuterated buffers is characteristic of peptides which have a helical structure. However, significant disorder in peptide chains is still present as evidenced by the large halfwidth of the amide I band (indicating a high degree of motional freedom of amide C=O groups) and the occurrence of a shoulder on the amide I band at 1644 cm^{-1}. This small increase in helical content agrees well with our CD results. The adoption of a partial α-helical structure is presumably the result of the neutralization of the positive charge on the peptide chain by the negatively charged sulphate headgroups. Porcine fragment 1–16 could also be shown to interact with SDS in a similar manner, resulting in the formation of a similar proportion of helical secondary structures (Fig. 4*b*). Rather surprisingly, the human galanin fragment 1–16 showed no interaction with SDS. This difference between the human and porcine fragments is difficult to rationalise considering the minor differences in sequence between the two peptides (porcine Ile16 to human Val16). The additional feature at 1672 cm^{-1} arises from the trifluoroacetate counterion commonly bound to synthetic peptides. In the presence of SDS, M35 and M15 exhibited spectra similar to those seen in aqueous solution.

Addition of porcine galanin, human or porcine fragments 1–16 and M35 to suspensions of the zwitterionic lipid DHPC

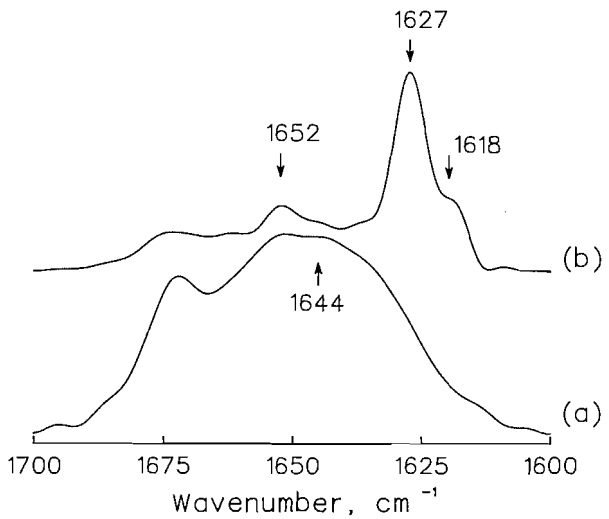

FIG. 5. FT-IR spectra in the amide I region of M15 in the presence at DHPC at 20°C (*a*) and 45°C (*b*).

resulted in spectra identical to those seen in aqueous solution, in agreement with our CD results obtained for rat galanin in the presence of DOPC. An amide I maximum centered at 1646 cm^{-1} (similar to that seen in Fig. 1) indicated that no interaction had occurred between the peptide and the lipid bilayer. On the other hand, the galanin antagonist M15 exhibited a weak band at 1652 cm^{-1} (in addition to the major band at 1644 cm^{-1}) in the presence of DHPC, indicative of the appearance of some helical structure (Fig. 5*a*). This may be attributed to the high content of hydrophobic amino acids in the sequence, which will favour penetration of the peptide into the hydrophobic bilayer core. Again, the band at 1672 cm^{-1} arises from trifluoroacetate contamination. Elevation of the temperature to 45°C (Fig. 5*b*) resulted in the appearance of a new band at 1627 cm^{-1}. This strong feature, typical of proteins which are aggregated following thermal or solvent denaturation (18), arises from the formation of intermolecular hydrogen bonds between amide C═O and N—H groups of peptide chains. Thermal denaturation of the peptide at the relatively low temperature of 45°C suggests that the membrane induced α-helix is unstable. In addition to the intense amide I band at 1627 cm^{-1} a weaker feature at 1618 cm^{-1} is apparent, a position similar to that seen for the side chain vibrations of tyrosine. However, the intensity of this absorption is greater than would be expected for a peptide of this size containing a single tyrosine residue. The additional intensity may be attributed to absorptions arising from a second class of intermolecular hydrogen bonds formed between peptide chains. The much lower frequency associated with this class of intermolecular hydrogen bonds indicates that it is extremely strong, suggesting close alignment of aggregated chains. The presence of two amide I bands indicative of aggregation is not unusual, and has been demonstrated for other biologically active peptides including magainins (19) and substance P (20).

In conclusion, studies such as that presented here highlight the utility of spectroscopic techniques in comparing the structural properties of biological peptides and their analogues, possibly as a first step in general screening for the design of novel therapeutic agents.

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