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NMR structure of the glucose-dependent insulinotropic polypeptide fragment, GIP(1–30)amide

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Abstract

Glucose-dependent insulinotropic polypeptide is an incretin hormone that stimulates insulin secretion and reduces postprandial glycaemic excursions. The glucose-dependent action of GIP on pancreatic β-cells has attracted attention towards its exploitation as a potential drug for type 2 diabetes. Use of NMR or X-ray crystallography is vital to determine the three-dimensional structure of the peptide. Therefore, to understand the basic structural requirements for the biological activity of GIP, the solution structure of the major biologically active fragment, GIP(1–30)amide, was investigated by proton NMR spectroscopy and molecular modelling. The structure is characterised by a full length α-helical conformation between residues F^6 and A^{28}. This structural information could play an important role in the design of therapeutic agents based upon GIP receptor agonists.

Keywords: GIP; Type 2 diabetes; Molecular modelling; NMR

Glucose-dependent insulinotropic polypeptide (GIP), also known as gastric inhibitory polypeptide, is a potent glucose-lowering gastrointestinal peptide hormone. GIP is considered to be one of the principal incretin factors of the enteroinsular axis, accounting for up to 80% of the insulin response to nutrient ingestion [1]. Other actions of GIP include metabolic effects on adipocytes and recently discovered links with obesity [2–4]. The GIP receptor is a seven transmembrane G-protein coupled receptor belonging to the family of secretin, glucagon, and VIP receptors. GIP receptor mRNA is widely distributed in peripheral organs, including the pancreas, gut, adipose tissue, heart, adrenal cortex, and the brain [5]. Human GIP consists of a single 42 amino acid polypeptide chain derived by proteolytic cleavage of the 153 residue precursor preproGIP, giving rise to a 51 amino acid N-terminal fragment, the 42 residue GIP in the middle section, and a 60 amino acid segment at the C-terminus.

Due to the insulinotropic and glucose-lowering properties of GIP, there is interest in developing novel GIP analogues for the treatment of diabetes mellitus [6]. Determination of the structural, biological, and functional properties of the peptide is vital in understanding structure–activity relationships. There have been many reports regarding pharmacological evaluations of GIP and its analogues [6–8]. Structure–function and conformational analyses carried out by circular dichroism (CD) spectroscopy were also reported [9]. However, to our knowledge, there is no NMR or X-ray crystallographic structural information available for GIP or its receptor.

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In this paper, we present the three-dimensional solution structure of GIP(1–30)amide (YAEGTFI SDYSIAMDKIHQQDFVNWLLAQK) in a 2,2,2-trifluoroethanol (TFE-d_3)–H_2O solvent mixture, as determined by NMR spectroscopy and molecular modelling. We show that GIP(1–30)amide retains the full bioactivity of the native GIP(1–42). These results will help to provide a valuable insight into the structure and function of the parent GIP(1–42). Our findings should also be useful in the design of novel GIP analogues and drugs active at the pancreatic GIP receptor.

Materials and methods

Peptide synthesis and characterisation by MALDI-TOF mass spectrometry. Human GIP(1–30)amide and native GIP(1–42) were sequentially synthesised on an Applied Biosystems automated peptide synthesiser (model 432A) using standard solid-phase Fmoc procedure, starting from an Fmoc-Gln-Wang resin as described previously [7]. The peptides were purified to homogeneity by reversed phase-HPLC and structural identity was confirmed by MALDI-TOF [10].

Insulin releasing activity. Cultured pancreatic BRIN-BD11 cells were employed to assess insulin releasing activity [11]. Test incubations were performed by 20 min incubation at 37 °C in 1.0 ml in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 0.5% (w/v) bovine serum albumin, 5.6 mmol/L glucose and supplemented with a range of concentrations (10^{-13}–10^{-6} mol/L) of GIP peptides. Insulin was determined by radioimmunoassay [12].

NMR spectroscopy. NMR experiments were performed using the 5 mm inverse probe head on Bruker DRX 500 and 900 NMR spectrometers operating at a ^{1}H resonance frequency of 500.13 and 900.27 MHz, respectively, at 298 K. The peptide sample (2 mM, pH 3.0 uncorrected) was dissolved in a 1:1 mixture of TFE-d_3 and H_2O (650 μl), except for the exchange studies where the peptide sample was dissolved in a 1:1 mixture of TFE-d_3 and D_2O.

One-dimensional (1D) ^{1}H NMR spectra and two-dimensional (2D) phase-sensitive double-quantum filtered correlation spectroscopy (DQF-COSY) [13], total correlation spectroscopy (TOCSY) [14], and nuclear Overhauser effect spectroscopy (NOESY) [15] data sets were acquired with a relaxation delay of 1.5 s, acquisition time of 0.146 s, and a spectral width of 10 kHz. The TOCSY data were acquired with 40 and 80 ms mixing times, whereas the NOESY data were acquired with a 200 ms mixing time. The experiments were performed with 32, 16, and 32 transients for each of 2K t_1 increments into 8K complex data points for the DQF-COSY, TOCSY, and NOESY data, respectively. 2D data were zero-filled to 4K data points in F_1 prior to transformation. All data sets were apodised in both dimensions using a shifted squared sinebell window function. Variable temperature studies were carried out using a standard 1D ^{1}H NMR pulse sequence. All spectra were referenced internally to the residual ^{1}H signal of $F_3CCD\underline{H}OD$, resonating at 3.98 ppm. Data were processed using the Bruker XWINNMR program (version 3.5).

Structure calculations. Spectra were analysed using the TRIAD module of Tripos software [16]. Structure calculations were carried out using the CYANA program (version 1.0.6) [17]. The NOESY data were used to generate upper and lower distance constraints, and to classify them according to the previously defined upper bounds of 2.8, 3.6, and 5.0 Å. The lower bound was set to the van der Waals radius of 1.8 Å. The threshold values of upper bounds were established based on sequential distances of d_{NN} and $d_{\alpha N}$ [18]. Pseudoatoms were also introduced [19]. Constraints for 8 hydrogen bonds (1.8–2.0 Å for H–O and 2.7–3.0 Å for N–O distances) were introduced for amide protons that showed slow exchange rates [20]. Torsional angle constraints in the −90° to −40° range were also included for $^3J_{HNH\alpha} < 5.5$ Hz.

Sixteen torsional angle constraints and 549 upper distance constraints obtained from the NOESY cross-peak volumes, including 16 constraints for hydrogen bonds, were used as input to CYANA. Distance constraints involving fixed distances as well as those limits that could not be violated were regarded as irrelevant by the program. Two hundred structures were randomly generated and energy minimised in CYANA, including 10,000 steps of simulated annealing, as well as 5000 steps of conjugate-gradient minimisation. During the CYANA calculations, distance and torsional constraints were weighted using the force constants $k_{NOE} = 1$ kJ mol^{-1} Å^{-2} and $k_{Dihed\_c} = 5$ kJ mol^{-1} deg^{-2}, respectively. Finally, the 20 structures with lowest target function values were subjected to 1000 steps of unconstrained Powell minimisation using the Tripos force field within SYBYL. MOLMOL [21] was used for structural descriptions.

Results and discussion

GIP(1–30)amide showed a similar dose-dependent insulinotropic pattern to native GIP(1–42) over the range 10^{-9}–10^{-6} M ($P < 0.01$) (Fig. 1). These observations are in broad agreement with the findings of the group of Pederson in Canada [9,22,23], indicating that GIP(1–30)amide is a good starting candidate for the spectroscopic and structural studies on the GIP family of peptides.

The NMR data obtained from the 500 MHz magnet were not sufficient to identify individual resonances due to partial signal overlap. Therefore, 900 MHz data were used for both resonance identifications and structure calculations. The NMR signals observed for GIP(1–30)amide in the aqueous TFE solvent were sharp and well resolved. Almost all the amino acid spin systems could be distinguished from one another in the TOCSY spectrum. The DQF-COSY spectrum was used for the identification of overlapping signals. Despite the low molecular mass of this peptide (~3 kDa), a large

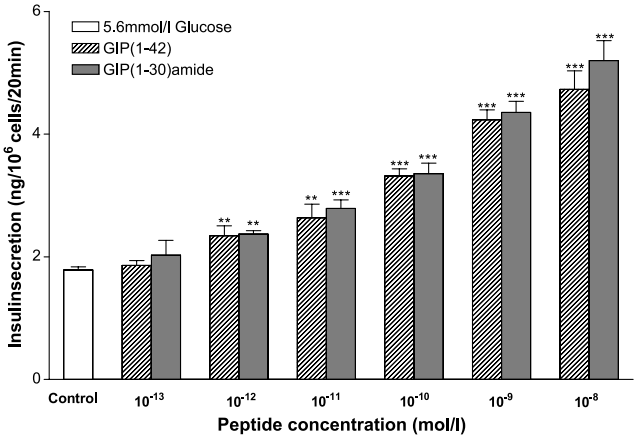

Fig. 1. Effects of native GIP(1–42) and GIP(1–30)amide on insulin secretion from BRIN-BD11 cells at 5.6 mmol/L glucose. Values are means ± SEM ($n = 6$) $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control (5.6 mmol/L glucose).

number of clear, sharp nuclear Overhauser effects (NOEs) were observed in the NOESY spectrum, a characteristic normally associated with folded proteins. Sequence-specific resonance assignments were carried out, which led to the full and unambiguous identification of all the individual spin systems. However, some side-chain and aromatic protons could not be assigned due to signal overlap. The NH–αH region of the NOESY spectrum and the NOE connectivities of GIP(1–30)amide are shown in Figs. 2 and 3, respectively. All the side chain spin connectivities through αH signals were clearly observed in the TOCSY spectrum for most of the amino acid residues. While some weak cross-peaks were observed for the methyl resonances of Ala, Val, and Leu residues in the TOCSY spectrum, these signals were clearly identified in the NOESY spectrum.

The presence of $d_{\beta N}(i,i+1)$ and $d_{NN}(i,i+1)$ connectivities of neighbouring amino acid residues supported the reliability of the resonance assignments. The fingerprint region of the NOESY spectrum showed clear medium-range $d_{\alpha N}(i,i+3)$ connectivities for most of the residues, thus revealing the occurrence of elements of secondary structure. Overlapping cross-peaks were observed for A^{13}/K^{16}, D^{15}/H^{18}, K^{16}/Q^{19}, and W^{25}/A^{28}. The aliphatic region of the NOESY spectrum showed $d_{\alpha\beta}(i,i+3)$ cross-peaks for most of the residues, thus supporting the presence of an α-helical segment between T^5 and A^{28}. The prominent $d_{\alpha N}(i,i+3)$, $d_{NN}(i,i+1)$, and $d_{\alpha\beta}(i,i+3)$ connectivity patterns, as well as small $^3J_{HNH\alpha}$ coupling constants (~4 Hz) measured from the 1D and 2D DQF-COSY NMR spectra, confirmed the α-helical character of GIP(1–30)amide.

The temperature dependence of amide protons was measured over the range 283–307 K. A structured state was apparent from the small temperature gradients ($\Delta\delta/\Delta T$) of −1.8, −3.1, and −2.9 ppb/C of residues T^5, D^{15}, and W^{25}, respectively. Temperature coefficients for all the other distinguishable amide protons were in the region of −4.0 ppb/C, indicating shielding of the amide protons of residues T^5–A^{28} from solvent exchange. This suggested that amide protons within the helical region were involved in hydrogen bonding. The

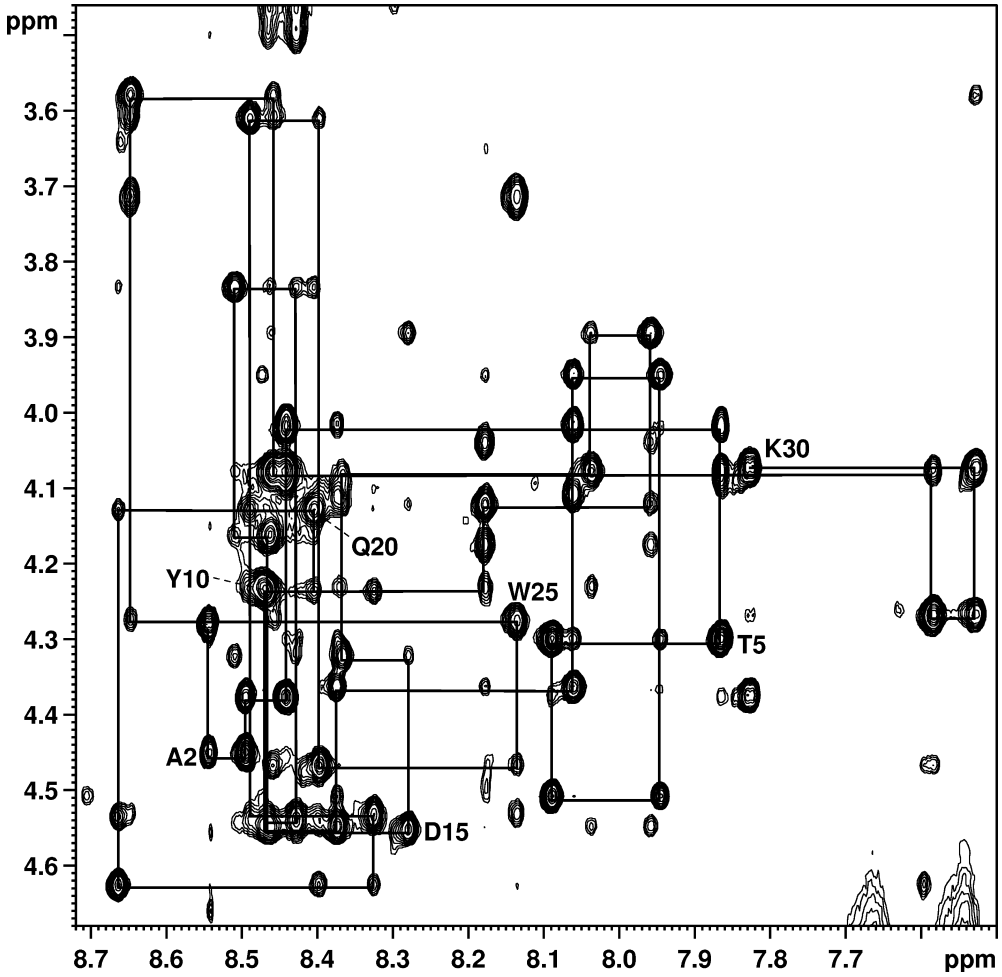

Fig. 2. Fingerprint (NH–αH) region of the 2D NOESY NMR spectrum of GIP(1–30)amide. The backbone 'walk' is indicated together with residue assignments.

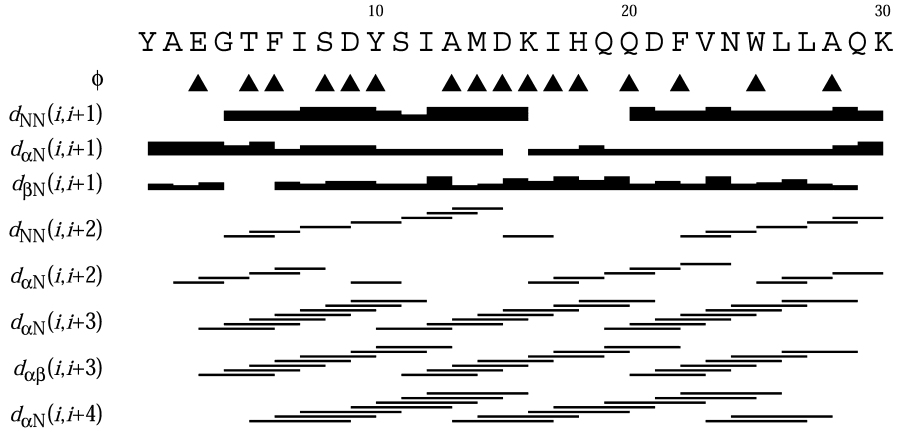

Fig. 3. Short- and medium-range NOE connectivities for GIP(1–30)amide.

H–D exchange studies of GIP(1–30)amide showed somewhat slower amide proton exchange rates for the residues I^{7}, S^{11}, I^{12}, A^{13}, H^{18}, F^{22}, W^{25}, and A^{28}. This is an indication that the amide protons have poor solvent accessibility. However, some exchanging protons could not be identified due to the signal overlap. These qualitative observations clearly support the α-helical character of GIP(1–30)amide in an aqueous TFE solvent mixture. Fig. 4 shows the bundle of 20 structures resulting from the combination of CYANA and the energy minimisation protocols. Analysis of the ensemble of 20 structures with PROCHECK-NMR [24] suggests an α-helical arrangement between residues F^{6} and A^{28}. The root-mean square deviation (RMSD) values for a best fit superposition between residues 6 and 28 of backbone and heavy atoms were 0.26 ± 0.06 Å and 1.06 ± 0.18 Å, respectively. All ϕ/ψ values for non-glycine residues fell into allowed (88%), additionally allowed (11%), and generously allowed (1%) regions in the Ramachandran plot (Table 1).

Generally, peptides of the same family that have similar sequences show similar structural features. Structural studies of peptide hormones similar to GIP, namely GLP-1 [25], exendin-4 [26], and glucagon [27], carried out in different media, have revealed that they all show α-helical features throughout the peptide sequence. Structural studies of exendin-4 show an αH chemical shift index (CSI) pattern [28] consistent with the presence of an α-helix between residues 7 and 27 [26]. Studies of GLP-1 in TFE also reveal an α-helical arrangement between residues 7 and 28, distinguishing two helical segments between residues 7–15 and 17–28 [25]. Unlike GLP-1, our studies of GIP(1–30)amide in aqueous TFE showed a continued αH CSI pattern, indicating that there is a continuous α-helix from residues 6 to 28. Comparison of our calculated structures of GIP(1–30)amide with solution structures of similar peptides deposited in the protein data bank (PDB) shows a remarkable structural resemblance of the helical regions.

Peptides and proteins are naturally formed in aqueous environments. The NMR data obtained for GIP(1–30)amide in water showed a mainly random coil conformation, being slightly folded towards the C-terminus. This phenomenon was also observed in studies of GLP-1 [25]. On the other hand, less polar solvents, such as TFE, tend to induce a more defined secondary structure in peptides because the lesser ability of these solvents to take part in hydrogen bonds with the peptide promotes the formation of internal hydrogen bonds. TFE has been used extensively as a co-solvent in the determination of solution structures of peptides by NMR spectroscopy. It has been shown that TFE promotes the formation and stabilisation of helical regions in peptides with an intrinsic helical propensity [29,30]. Other solvents, such as methanol, have also been used in NMR structural studies of peptides [31]. As a result of using this type of solvent, structures tend to be more ordered in comparison with the random coil or slightly folded features commonly found for such short peptides when dissolved in pure water.

Studies have been carried out on the biological relevance of the N-terminal region of GIP and related peptides [9,32]. However, most of them have been unable to describe the structure of this region in detail. Our studies have also failed to reveal any defined structural features for the residues at the beginning of the N-terminus. The CD, NMR, and X-ray structural studies of GIP related peptide hormones such as GLP-1, exendin-4, and glucagon, showed a helical structure as the preferred conformational state [25–27]. CD spectroscopic studies have shown that the helical content of GIP(1–30)amide in 20 mM phosphate buffer was 8.5%, whereas the helicity of GIP and GIP(1–30)amide in 20 mM phosphate buffer at pH 7, containing 40% TFE, was 79% and 45%, respectively [9]. However, some CD studies of GIP fragments suggest contradictory secondary structural correlations. This phenomenon is not uncommon in the analysis of small peptide fragments by CD spectroscopy.

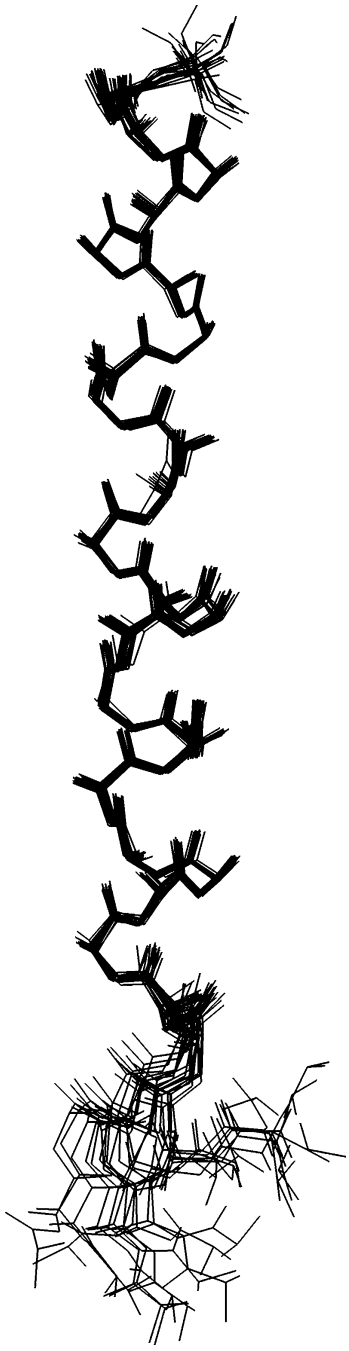

Fig. 4. Best fit superposition of the 20 selected structures of GIP(1–30)amide, between residues 6 and 28. Only bonds between the backbone atoms are shown. N-termini are shown at the bottom.

In conclusion, our studies show that in a 50% aqueous TFE solvent, GIP(1–30)amide has an α-helical structural region from F^6 to A^{28}. The structures calculated for GIP(1–30)amide remain within one family of conformations and the level of agreement between the structures demonstrated the ordered arrangement. The 50% aqueous TFE solvent mixture proved to be essential in this research, since results obtained from pure water did not produce enough information to carry out structural studies. The fact that GIP(1–30)amide has been dissolved in an aqueous TFE solvent may call into question the biological relevance of the structures obtained in this study. Nonetheless, the results presented in this paper form the basis for further biological and structure–activity relationship studies of GIP and may lead to the development of therapeutic analogues for the treatment of diabetes. Biological and NMR studies of GIP and its analogues are in progress and will be published elsewhere.

Table 1
Structural statistics of GIP(1–30)amide in 50% aqueous TFE

Total NOE constraints	533
Total H-bond constraints	16
Torsional constraints	16
NOE violations > 0.2 Å	23
Ramachandran plot regions (%)	
Favoured	88
Additionally allowed	11
Generously allowed	1
Mean atomic rmsd (Å)	
Backbone atoms (residues 6–28)	0.26 ± 0.06
Heavy atoms (residues 6–28)	1.06 ± 0.18
Average energies (kcallmol)	
Bond stretching	17.142
Angle bending	74.515
Torsional	56.366
OOP bending	0.404
1–4 van der Waals	35.704
van der Waals	−168.900
Total	15.230

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