Glucose-dependent incretin hormone GIP (1) is synthesized and secreted in the gut and acts as an incretin hormone to stimulate insulin secretion and promote glucose-lowering action (2). GIP is also involved in the regulation of food intake and energy metabolism (3). GIP is known to stimulate insulin secretion, promote glucose-stimulated insulin secretion, and play a role in the regulation of fat metabolism (4). GIP has been shown to be involved in the regulation of energy metabolism and glucose homeostasis (5). GIP is a key player in the physiological control of glucose and energy metabolism (6). GIP is also involved in the regulation of food intake and energy metabolism (7). GIP has been shown to be involved in the regulation of energy metabolism and glucose homeostasis (8). GIP is a key player in the physiological control of glucose and energy metabolism (9). GIP is also involved in the regulation of food intake and energy metabolism (10). GIP has been shown to be involved in the regulation of energy metabolism and glucose homeostasis (11). GIP is a key player in the physiological control of glucose and energy metabolism (12).
carried out with the program K2D (22) using the DICROWEB web interface (23–25).

**NMR Spectroscopy**—NMR experiments were performed on Bruker DRX 500, 800, and 900 NMR spectrometers equipped with 5-mm inverse probe heads, operating at 298 K and at proton resonance frequencies of 500.13, 800.13, and 900.27 MHz, respectively. An 2 mM peptide sample was prepared using a 9:1 mixture of H$_2$O/D$_2$O (600 μl, pH 3.3, uncorrected). Two-dimensional phase-sensitive DQF-COSY (26), TOCSY (27), and NOESY (28) spectra were acquired with a relaxation delay of 1.4 s, an acquisition time of 0.40 s, and a spectral width of 10 kHz. TOCSY spectra were acquired with 30- and 80-ms mixing times. NOESY spectra were acquired with mixing times of 200 ms. All DQF-COSY, TOCSY, and NOESY experiments were performed with 16, 8, and 16 transients for each of 2,048 $t_1$ increments into 8,192 complex data points, respectively. All spectra were zero-filled to 4,096 data points in $F_1$ and apodized using a shifted squared sinebell window function in both dimensions. The signal of 3-(trimethylsilyl)propionic acid was used as an internal chemical shift reference at 0.0 ppm. All data were acquired and processed using Bruker XWINNMR (version 3.5).

**Structure Calculations**—Two-dimensional NMR spectra were analyzed with SPARKY (version 3.110) (29). The integrals of the NOEs were converted into distance constraints with CALIBA (30) according to three proton classes, namely backbone, flexible side chain, and methyl protons. Pseudatoms were also introduced for protons that could not be stereospecifically assigned (31). Structure calculations were carried out using CYANA (version 1.0.6) (30). 200 random structures were generated, and their energy was minimized using the anneal protocol in CYANA, including 10,000 steps of simulated annealing, as well as 5,000 steps of conjugate-gradient minimization. During the CYANA calculations, distance constraints were weighted using the force constant $k_{NOE} = 1$ kJ mol$^{-1}$ Å$^{-2}$. The 20 structures with lowest target function values were subjected to 5,000 steps of unconstrained Powell minimization with SYBYL (version 6.8.1) using the Tripos SYBYL Force Field (32). MOLMOL (33) and PROCHECK-NMR (34) were used for further structure analysis.

**Back Calculation**—One of the low energy GIP models from the final 20 conformations and the individual peak positions and their assignments from the NOE experiment with a mixing time of 200 ms were used to simulate the fingerprint region of the NOE spectrum using the CORMA module (35) within SYBYL. The calculation was performed using a model-free approach, overall correlation times of 1–10 ns, and a Gaussian line shape. A fixed normalization method was also used along with the three-site methyl jump model to calculate methyl distances. Further CORMA simulations were carried out for the five lowest energy conformations.

**RESULTS**

Fig. 1 shows the effects of GIP and GIP peptides on insulin secretion, from clonal pancreatic BRIN-BD11 cells. From this figure, it is clear that native GIP dose-dependently (10$^{-12}$–10$^{-6}$ M) stimulated insulin secretion when compared with control incubations (5.6 mM glucose alone) ($p < 0.001$). Similarly, GIP-(1–42)Ala$^6$ also stimulated insulin secretion when compared with control ($p < 0.01$ to $p < 0.001$), however not to the same extent as native GIP ($p < 0.05$ to $p < 0.01$). All other GIP peptides displayed decreased insulinotropic activity (1.2–1.9-fold decrease; $p < 0.05$ to $p < 0.001$) when compared with both glucose control and GIP.

The influence of pH on the secondary structure of GIP was studied by means of CD spectroscopy. 30 μM GIP samples in 20 mM acetate buffer (pH 4.0, 5.0) and phosphate buffer (pH 6.0, 7.0, 8.0) were analyzed in the far-UV (Fig. 2A), as well as an unbuffered 30 μM sample of GIP in water (pH 6.8, uncorrected) (Fig. 2B). The helical content of GIP gradually increased from 10 to 26%, from pH 8.0 to 4.0, respectively. The helical content of the buffered sample at pH 7.0 was 20%, whereas that of the unbuffered sample was 7%.

One- and two-dimensional NMR data were obtained using 500-, 800-, and 900-MHz spectrometers. The DQF-COSY and TOCSY spectra provided a basis for the identification of individual residue spin systems. Except for the N-terminal residue Tyr$^1$, all other resonances were clearly observed in the TOCSY spectrum. The chemical shifts of methyl resonances were used to identify the alanine, isoleucine, and leucine residues, as well as a unique valine residue. The 2 serine residues, Ser$^8$ and Ser$^{11}$, were identified by their distinctive βH proton resonances. Other spin systems could not be distinguished due to the similarity of their AMX spin pattern. Discrimination of non-unique residues and identification of all other unidentified spin systems was achieved by direct comparison of the TOCSY and NOESY spectra. The 2 glycine residues, Gly$^9$ and Gly$^{31}$, showed strong, prominent cross-peaks in the TOCSY and NOESY spectra. Alanine, glycine, isoleucine, serine, threonine, and valine residues were used as starting points for the identification of sequence-specific resonance assignments. The connectivities of Ala$^7$ to Asp$^9$ were clearly identified using the $\alpha$H/NH$_{\alpha}$, H and βH/NH$_{\beta}$ cross-peaks in the fingerprint region of the NOE spectrum (Fig. 3). Distinctive βH/NH intra-residue and methyl connectivities were used to identify the connectivities of Ser$^{13}$/Ile$^{14}$, Ile$^{15}$/Ala$^{16}$, Met$^{18}$/Asp$^{19}$, and Lys$^{17}$/Ile$^{17}$. The 2 glutamine residues, Gln$^{19}$ and Gln$^{20}$, were identified using the backbone and βH/NH connectivities of Asp$^{21}$.
were used to identify Gln\textsuperscript{29} and Lys\textsuperscript{32}, respectively. Identification of the Asn\textsuperscript{34}, Asp\textsuperscript{35}, and Trp\textsuperscript{36} spin systems was made via the backbone and $\beta$H/$\mathrm{NH}_{\beta+1}$ resonance connectivities of their neighboring residues. Aromatic and side-chain protons to inter-residue $\alpha$H and $\beta$H cross-peaks were observed for Trp\textsuperscript{36} and His\textsuperscript{35}. The prominent methyl resonances of Ile\textsuperscript{30} and Thr\textsuperscript{41} were used to identify the C-terminal end of the polypeptide chain. Sequence-specific resonance assignments were completed by identification of backbone, $\alpha$H, $\beta$H, aromatic, and side-chain resonance connectivities between immediately neighboring residues.

Other parts of the NOESY spectrum were then searched for secondary structure correlations. Strong $\alpha$H/$\beta$H medium range connectivities were observed suggesting the presence of an $\alpha$-helix for residues Ser\textsuperscript{11}–Gln\textsuperscript{29}, except those between Ala\textsuperscript{13}/Lys\textsuperscript{16}, Asp\textsuperscript{15}/His\textsuperscript{18}, His\textsuperscript{18}/Asp\textsuperscript{21}, Asp\textsuperscript{21}/Asn\textsuperscript{24}, due to chemical shift degeneracy or signal overlap. Clear medium range $\alpha$H/$\mathrm{NH}_{\alpha+1}$H connectivities for residues between Ser\textsuperscript{11} and Lys\textsuperscript{30} were also identified, except between Ala\textsuperscript{13}/Lys\textsuperscript{16} and Gln\textsuperscript{20}/Val\textsuperscript{23}, due to overlapping. Distance constraints derived from the NOESY data were used for structure calculations (Fig. 4). 200 random structures were generated using the CYANA protocol, and the 20 conformations that best fit the experimental constraints were subjected to additional unconstrained energy minimization. The main structural feature of the peptide is a right-handed $\alpha$-helix between residues Ser\textsuperscript{11} and Gln\textsuperscript{29}, as determined by MOLMOL and PROCHECK-NMR. Best fit superposition of the backbone atoms between residues Ser\textsuperscript{11} and Gln\textsuperscript{29} of the 20 calculated structures is presented in Fig. 5.

Simulated spectra were obtained for the five conformations with the lowest energy from the final set of 20 energy-minimized conformations, with overall correlation time values of 1–10 ns. Only the fingerprint region of the NOESY spectrum was simulated and compared with the experimental spectrum. The spectrum simulated with a correlation time of 2 ns showed the highest level of agreement with the experimental results (Fig. 6).

**DISCUSSION**

GIP-(1–42) is a known stimulator of insulin secretion in the presence of glucose (36). In this study, seven synthetic peptides with a single-residue replacement with an alanine in each of the first seven positions were synthesized to evaluate specific residues that play a role in insulin secretion. In comparison with GIP-(1–42), GIP-(1–42)Ala\textsuperscript{6} was the only analogue to show insulin-secreting activity similar to that of the native peptide ($p < 0.001$) when compared with glucose control; however, its insulinotropic ability was noticeably reduced. When compared against glucose control, the remaining alanine scan peptides (GIP-(1–42)Ala\textsuperscript{1}, GIP-(1–42)Ala\textsuperscript{3}, GIP-(1–42)Ala\textsuperscript{4}, GIP-(1–42)Ala\textsuperscript{5}, and GIP-(1–42)Ala\textsuperscript{7}) showed reduced or no insulinotropic activity, leading us to believe that the amino acid residues replaced are in fact vital to the insulin-releasing activity of GIP and that the N terminus is of great importance in glucose-dependent insulin release. Interestingly, GIP-(1–42)Ala\textsuperscript{3} and GIP-(1–42)Ala\textsuperscript{4} showed such a reduced level of insulinotropic activity that these may be considered for antagonistic studies in the future, especially since it is well known that GIP-(3–42) is a GIP-receptor antagonist (10). With such an inactivation of insulin release when glutamic acid in position 3 is replaced, it may suggest further that position 3 is of great importance in the insulinotropic potency of GIP. A sample of native GIP was also subjected to basic solubility studies. The peptide was solubilized in Tris buffer (10 mM Tris, 100 mM NaCl, 5 mM MgCl\textsubscript{2}, and 1 mM dithiothreitol, pH 7.5) and analyzed by spectrophotometry combined with light scattering. The results showed a single UV peak at 280 nm, revealing that GIP is monomeric under these conditions.
As can be seen from Fig. 2A, the CD spectra obtained in the far-UV at pH values 4.0–8.0 are characteristic of samples with some degree of helical secondary structure, whereas the spectrum acquired for the unbuffered sample (Fig. 2B) suggests an irregular secondary structure (37). Analysis of the spectra with the program K2D reveals an increase in helical content with decreasing pH. We have also found that GIP is 20% helical in 20 mM phosphate buffer, pH 7.0, increasing up to 26% when in 20 mM acetate buffer, pH 4.0. These results are in contrast with studies of Manhart et al. (38), in which they reported a helical content of 11% for GIP in 20 mM phosphate buffer, pH 7. Studies in the near-UV (results not shown), carried out for 0.95 mM GIP samples in phosphate buffer (20 mM, pH 7.0) and unbuffered (pH 3.0, uncorrected), show spectra similar to those found for folded proteins (37). The results could be interpreted as self-association, the adoption of a regular tertiary structure, or a combination of both. However, as our spectrophotometric and light-scattering experiments show that GIP is monomeric, then the CD results should reflect the adoption of a regular tertiary structure by GIP. The near-UV CD results could be valuable in future studies as a template of the tertiary structure of native GIP (39).
The NMR data obtained with the 800- and 900-MHz spectrometers were used for resonance identification and structure calculation. Two-dimensional DQF-COSY and TOCSY spectra were used for spin system identification. TOCSY and NOESY data were used for full and unambiguous assignment of individual and sequence-specific resonances. Some side-chain and most of the aromatic protons could not be assigned due to signal overlap. Identification of $\beta$H/NH, $N_i$H/$N_{i+1}$H cross-peaks, and aromatic proton connectivities to neighboring residues supported the reliability of the sequence-specific resonance assignments. The large number of well-spread NOEs observed suggested that GIP is in a folded state. The presence of $\alpha_i$H/$N_{i+3}$H NOE connectivities in the fingerprint region of the NOESY spectrum and of stronger $\alpha_i$H/$\beta_{i+3}$H NOEs in the range of Tyr$^{19}$–Gln$^{29}$ revealed the occurrence of elements of $\alpha$-helical secondary structure.

The helical character of GIP in water is further supported by the chemical shift index (40), which suggested the presence of two helical segments between residues Ser$^8$–Lys$^{16}$ and Gln$^{19}$–Lys$^{30}$. It was noted that the chemical shifts of all the isoleucine residues did not follow the predicted chemical shift index pattern. However, the absence of medium range $\alpha_i$H/$N_{i+3}$H and $\alpha_i$H/$\beta_{i+3}$H connectivities in the NOESY spectrum in the range Ser$^8$–Tyr$^{10}$, and their presence between residues Lys$^{16}$ and Gln$^{19}$, leads us to suggest a continuous $\alpha$-helical segment between residues 11 and 29. In addition, all of the calculated structures showed a continuous helical segment in this region of the peptide chain.

Analysis of the calculated structures revealed that the GIP in water adopts an $\alpha$-helical motif between residues Ser$^1$ and Gln$^{30}$ for most of the calculated conformations. These results were confirmed by the secondary structure predicted within the MOLMOL package. Further analysis of the structures with PROCHECK-NMR showed that more than 98% of the residues lie within allowed regions of the Ramachandran plot (Table 1).

To assess the quality of the calculated structures, the fingerprint region of the NOESY data was simulated and compared with the experimentally obtained spectrum. Simulated spectra showed an optimum level of agreement when a correlation time of 2 ns was used (Fig. 6). The fingerprint region of the simulated spectrum showed few additional cross-peaks with varying intensities. These peaks are mainly $\alpha_i$H/$N_{i+4}$H connectivities that arise from protons of residues that are part of the helical segment, such as $\alpha_{17}$H/$N_{21}$H, and $\alpha_{20}$H/$N_{24}$H. Also, several $\alpha_i$H/$N_{i+3}$H medium range connectivities from residues within the helical

| TABLE 1 |
|------------------|------------------|
| Structural statistics of GIP in water |
| NOE constraints used for calculations | 450 |
| NOE violations $>0.2$ Å | 23 |
| Ramachandran plot regions (%) |
| Most favored | 59.3 |
| Allowed | 33.9 |
| Generously allowed | 5.0 |
| Mean atomic r.m.s.d.$^*$ (Å) |
| Backbone atoms (residues 11–29) | $0.67 \pm 0.26$ |
| Heavy atoms (residues 11–29) | $1.53 \pm 0.33$ |
| Average energies (kcal mol$^{-1}$) |
| Bond stretching | 14.799 |
| Angle bending | 122.829 |
| Torsional | 75.199 |
| Out of plane bending | 0.905 |
| 1–4 Van der Waals | 47.870 |
| Van der Waals | $-203.511$ |
| Total | 58.091 |

$r.m.s.d.$, root mean square deviation.
region appeared stronger in the simulation, whereas αH/ναH cross-peaks were weaker. This could be due to the fact that CORMA calculates NOE intensities by assuming a rigid backbone structure. Therefore, the simulated spectrum showed weak connectivities, such as NOE intensities by assuming a rigid backbone structure. There-
tideinwater.
confirms that the calculated structures shows a true picture of the pep-
tstructure used for the simulation satisfies the experimentally derived
distance constraints; hence, the NOESY peak assignments and distance
constraint calibration were also accurately carried out. These results
confirmed that the calculated structure shows a true picture of the pep-
tide in water.

The root mean square deviation value of 0.67 ± 0.26 Å obtained after
superposition of the backbone atoms between residues Ser11 and Gln29
could suggest that the helical segment of the peptide chain is somewhat
flexible under these conditions. This may be due in part to the high
mobility of both the N-terminal and C-terminal ends at both sides of the
helix. This was supported by the lack of strong NOEs observed beyond the
N- and C-terminal ends of the helical region. However, NOEs observed
at the C-terminal end of the helix are marginally stronger than
those of the N-terminal end. In general, short peptides adopt mainly
random coil conformations when dissolved in pure water. In contrast,
we observed 10 NOEs per residue, which is much higher than the aver-
age of seven NOEs generally observed for peptides of similar size.

Ongoing studies in our laboratory showed that the biologically active
GIP fragment, GIP-(1–30)amide (16) and the parent GIP4 adopted a
tighter and better defined helical conformation when TFE was
introduced in the sample. This leads us to suggest that the lack of helix-
induced reverse-stabilizing solvents, such as TFE, trifluoroacetic acid, and
methanol, results in the solution structure of GIP in water being somewhat
less ordered. As a result, the lower number of short and medium
range NOE connectivities observed in the NOESY spectrum, in com-
parison with the GIP-(1–30)amide and GIP in TFE, contributes to the
lower precision of the resulting structures and slightly higher average
ergies (Table 1).

It seems obvious to attempt a direct comparison of the solution struc-
ture of GIP with that of the other major incretin hormone GLP-1. How-
ever, we believe that, in this case, the length of the peptide chain plays a
relevant role toward the adoption by these peptides of regular secondary
structural features in a purely aqueous solvent. On the basis of NMR
studies, the 29-amino-acid residue GLP-1 has been shown to adopt a
random coil conformation in water (15), as well as the 30-residue GIP
fragment, GIP-(1–30)amide (16). It was also noted that the formation of
the secondary structure was observed to start at the C-terminal end to
then spread toward the N terminus by the addition of helix-favoring
solvents. On the other hand, exendin-4, a 39-residue agonist of GLP-1
that shows a 50% sequence homology to GIP, adopts a helical confor-
mation in aqueous medium (41). Therefore, we believe that the residues
at the C-terminal end (Gly31–Gln39) of GIP appear to provide significant
stability to its structure in neat aqueous solvent. However, the C-termi-
nal segment does not seem to be important for insulinoctropic activity
since GIP-(1–30)amide shows a similar insulin-releasing activity to that
of the parent GIP (16). On the other hand, the N-terminal end, which
appears to be important for the activity of the peptide, remains disor-
dered under these conditions.

The proposed NMR structures are in good agreement with the results
of our CD spectroscopic studies. In view of the CD results, we may
conclude that the secondary structure of GIP depends strongly on the
buffering capacity of the solvent. The helical content of GIP increases
with decreasing pH. Since the sample studied by NMR was unbuffered
and its uncorrected pH was 3.0, we can also conclude that, at higher
concentrations, GIP acts as its own buffer, resulting in a more helical
conformation, as shown in our NMR studies.

In conclusion, we have shown that GIP adopts an α-helical confor-
mation between residues Ser11 and Gln29 when dissolved in pure water.
All 20 structures agree with the experimentally derived NMR con-
trast as indicated by the low number of NOE violations. These results
may help to gain further insight into the biologically relevant receptor-
bound structure of GIP and so facilitate the design of new analogues and
peptidic drugs to treat type 2 diabetes and obesity.

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