Solution structures of calcitonin-gene-related-peptide analogues of calcitonin-gene-related peptide and amylin

Julia A. M. HUBBARD,*§ Stephen R. MARTIN,† Lesley C. CHAPLIN,* Christopher BOSE,* Sharon M. KELLY‡ and Nicholas C. PRICE‡

*Celltech Ltd., 216 Bath Road, Slough, Berks. SL1 4EN, U.K., †Division of Physical Biochemistry, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K., and ‡Department of Biological and Molecular Sciences, University of Stirling, Stirling FK9 4LA, Scotland, U.K.

Near-u.v. and far-u.v. c.d. spectra of human α-calcitonin-gene-related peptide (hαCGRP), analogues and fragments of CGRP and amylin were recorded in aqueous solution and in trifluoroethanol (TFE)/water mixtures. All peptides contained significant amounts of α-helix in aqueous solution, and this amount increased on adding TFE. The helical content was unaffected by pH and salt. However, amylin contained much less helix than CGRP and the c.d. spectrum was more temperature-sensitive. A band in the near-u.v. c.d. spectrum of CGRP (but not present in the spectrum of amylin) was attributed to the disulphide bond in CGRP. The intensity of this band was pH-dependent and titrated with a pKₐ of 6.5, suggesting the involvement of histidine ionization.

INTRODUCTION

Human calcitonin-gene-related peptide (hCGRP) is a 37-amino acid-residue peptide that occurs in two forms, α and β, with the β form differing from the α form at three positions (Steenberg et al., 1985). The peptides have potent vasodilatory (Brain et al., 1985), cardioexcitatory (Etienne et al., 1984) and plasma Ca²⁺ effects (Tippins et al., 1984).

In order to understand the way in which CGRP interacts with its receptor it is clearly important to have information on the secondary structure of the peptide. In the present work the structure of hαCGRP and some of its analogues were studied by c.d. spectroscopy. We also studied CGRP-(8-37)-peptide, a C-terminal fragment that behaves as a competitive antagonist against the intact peptide (Chiba et al., 1989). Amylin is a related 37-amino acid-residue peptide that shows 43% sequence identity with hαCGRP (Cooper & Greene, 1989). It has been demonstrated that both peptides are potent inhibitors of glycogen synthesis in rat skeletal muscle in vitro (Leighton & Cooper, 1988). It has also been suggested that in type 2 diabetes mellitus there may be sufficiently high concentrations of amylin to interact with the CGRP effector system (Leighton & Cooper, 1988). Therefore we were interested in seeing if amylin and hαCGRP share common structural features. Previous results obtained with use of c.d. (Manning, 1989) were interpreted as showing the presence of 20% helix in aqueous solution and up to 60% helix in 1:1 (v/v) TFE/water mixtures. In contrast, Breeze et al. (1991) concluded from n.m.r. spectroscopy that the peptide was unstructured in aqueous solution and formed only 27% helix in 1:1 (v/v) TFE/water. The present paper also describes experiments that attempt to resolve these differences.

MATERIALS AND METHODS

Peptides

The sequences of the peptides used are shown in Fig. 1. Synthetic human α-CGRP and human amylin were purchased from Bachem Feinchemikallen (Bubendorf, Switzerland) and Peninsula Laboratories (Belmont, CA, U.S.A.) respectively. The CGRP analogues [Asn²,Ile₂, Ser³]CGRP, [Asn²,Asp⁴, Phe⁶, Gly⁹³]CGRP, [Asn², Ser⁶⁺]CGRP and CGRP-(8-37)-peptide were synthesized in-house by solid-phase techniques with an Applied Biosystems 430A peptide synthesizer employing fluoren-9-ylmethoxycarbonyl/t-butyl chemistry and the Rink resin. The peptides were deprotected with trifluoroacetic acid and purified by gel filtration and preparative reverse-phase h.p.l.c. Amino acid analysis after acid hydrolysis gave the expected molar proportions. Fast-atom-bombardment m.s. gave molecular ions in agreement with calculated values.

C.d. spectroscopy

C.d. spectra were recorded from 260 to 197 nm and from 350 to 255 nm with a JASCO J600 spectropolarimeter for a range of protein concentrations (9.0 to 0.08 mg/ml) and pathlengths (0.1 to 40 mm) in increasing amounts of trifluoroethanol (TFE) (0 to 50%, v/v). The effect of pH was examined between pH 3.5 and pH 9.0 in water or buffer (20 mm-sodium phosphate). Temperature was varied between 18 °C and 50 °C. The concentration of peptides was determined by using the absorbance at 215 nm for CGRP (Manning, 1989) and amino acid analysis.

The content of secondary structure was obtained by using the CONTIN program of Provencher & Glöckner (1981), and data analysis of pKₐ values was carried out with Enzfitter software from Elsevier.

RESULTS AND DISCUSSION

Conformation in aqueous solution

Fig. 2 shows the u.v. c.d. spectra of hαCGRP under various solution conditions. The spectra show an intense negative band at approx. 200 nm and a broad shoulder at longer wavelengths. This band shape indicates that the peptide is largely unstructured.
but does contain some non-random structure, most probably α-helix. This interpretation was confirmed by analysis of the c.d.-spectra for secondary structure with the CONTIN program (Provencher & Glöckner, 1981). The helix content (about 20%) at 20 °C is independent of pH between pH 3.5 and pH 9.0 and buffer (20 mM-phosphate). This confirms the result obtained at pH 7.7 by Manning (1989) but is at variance with the n.m.r. data at pH 3.5 (Breeze et al., 1991). The secondary structure was unaffected by pH, and the spectra were identical over the pH range 3.5–9.0 (Fig. 2) both in the presence and in the absence of salt (20 mM-sodium phosphate). Increasing the temperature from 18 to 50 °C resulted in decreased helix content from 20% helix at 18 °C to 12–14% helix at 50 °C (Table 1). Amylin contained significantly less helix than hzCGRP in aqueous solution, and the secondary structure was also affected more by temperature (Fig. 3). The helix content of amylin at pH 7.0 decreased from 13% at 25 °C to about 3% at 50 °C (Table 1).

Conformation in TFE/water mixtures

Increasing the TFE content in solution resulted in a dramatic increase in the helix content of hzCGRP (Fig. 2), as shown by the increase in the intensity at 222 nm. The helix content increased from 20% at 0% TFE to 70% at 50% (v/v) TFE, independent of pH (over the range pH 3.5–7.0) or buffer (20 mM-sodium phosphate). Therefore the helix content in 50% TFE determined by c.d. is greater than that determined by n.m.r. under all conditions studied. The presence of an isodichroic point at about 202 nm indicates that the TFE-induced transition is largely between two conformations, most probably α-helix and random coil.

Increasing TFE concentrations also resulted in an increase in the helix content in amylin, as shown in Fig. 3, although the increase was much smaller than that observed with hzCGRP: the helix content in amylin at pH 7.0 rose from 13% at 0% TFE to 20% at 50% TFE (Table 1). However, in the presence of SDS (0.4%, in 20 mM-sodium phosphate buffer, pH 7.0, at 25 °C) a high helix content (approx 62%) was observed for amylin, similar to that observed previously for hzCGRP (approx. 60%) by Manning (1989).

Near-u.v. c.d. spectra

Fig. 4 shows the near-u.v. c.d. spectra of hzCGRP in aqueous
solution and in 50% TFE. The spectra show a broad absorbance centred around 280–290 nm, which we attribute to the disulphide bond between residues 2 and 7 (Strickland, 1984) as it was eliminated by adding dithiothreitol (25 mM) and was absent from the spectrum of the haCGRP-(8–37)-peptide fragment (Fig. 4). The data of Fig. 2 show that the secondary structure of CGRP is not affected by pH. In contrast, the spectra in Fig. 4 demonstrate that decreasing the pH from 7.76 to 3.15 results in a 3-fold increase in the dichroic absorption attributed to the disulphide bond. This suggests that the peptide is undergoing a pH-dependent change in tertiary structure. Fig. 5 shows a plot of Δ\(\Lambda\)\(_{222}\) against pH. These data yield an apparent pK\(_a\) of 6.5 when analysed by Enzfit software. This is consistent with the expected pK\(_a\) of histidine at residue 10 in haCGRP, which is close to the disulphide bond at residues 2 and 7. Similar results were obtained in the absence of TFE. In contrast with haCGRP, the disulphide bond in amylin does not exhibit c.d. in the near-u.v. region.

**Effects of protein concentration**

A concentration-dependent decrease in the amount of α-helix was observed above about 10 \(\mu\)M-haCGRP in aqueous solution; the helix contents at 10 \(\mu\)M and 1.7 \(\mu\)M were 19% and 8% respectively. Aggregation in these samples was not detected by light-scattering in absorption spectra recorded from 400 nm. However, such measurements would not have detected small-scale self-association such as that which occurs with calcitonin analogues of haCGRP, in which a monomer/trimer equilibrium occurs above 0.42 \(\mu\)M (Moe & Kaiser, 1985). These results may explain the discrepancy between the n.m.r. and c.d. data, as the n.m.r. experiments are carried out at high peptide concentrations (10–18 nm), when the peptide is less structured.

**Conformation of analogues of haCGRP**

The helix content of the analogues of haCGRP (Fig. 1) in aqueous solution and for a range of TFE concentrations is shown in Fig. 6. In aqueous solution the structure of the analogues appeared to be similar to that of haCGRP, except for the CGRP-(8–37)-peptide, which contained significantly lower amounts of helix (Fig. 6). The CGRP-(1–7)-peptide fragment does not exhibit c.d. at 222 nm (results not shown). These results suggest that the presence of the N-terminal region stabilizes the structure of the remainder of the peptide. For all the analogues investigated the helix content increased with increasing TFE.
concentrations. However, [Asn³, Asp¹⁴, Phe¹⁵, Gly²⁸³]CGRP had lower helix content (47%) than haCGRP in 50% TFE. As this analogue had similar biological activity to haCGRP, it suggests that formation of the high amounts of helix observed in haCGRP are not necessarily essential for receptor binding and activity. It has recently been demonstrated that N-terminal fragments of CGRP (e.g. CGRP(1–15)-peptide) have activity although compared with the whole peptide their potency is reduced (Maggi et al., 1990).

General discussion

Human amylin and haCGRP have an overall sequence identity of about 40%. Structure prediction methods (Sawyer et al., 1988) indicate that the peptides are expected to exhibit similar structures; the strongest potential to form helix lies from residues 9 to 16 in amylin and from residues 8 to 16 in CGRP, although Lynch & Kaiser (1988) suggested that the potential to form a helix extends to residue 25 in CGRP. There is a similar propensity for β-turn structure in the two peptides: residues 17–21 and 29–34 in haCGRP and residues 19–23 and 29–34 in amylin. However, there is a greater tendency for β-sheet structure in haCGRP (resides 4–11) than in amylin.

Nevertheless, the two peptides form very different structures both in aqueous solution and TFE/water mixtures. Amylin contains 20% helix in 50% TFE whereas CGRP contains 45–70% helix. The haCGRP contains 25% more helix than predicted, which was also observed in rat CGRP (Lynch & Kaiser, 1988). The structure of amylin was also more sensitive to temperature than CGRP.

Amylin exhibited CGRP activity, but was found to be 100 times less potent than CGRP, and the durations of the effects of amylin were much shorter than those of haCGRP. Amylin was also inhibited by the CGRP antagonist CGRP-(8–37)-peptide. It is noteworthy that the biological properties of haCGRP-(1–15)-peptide of reduced potency and duration of effects compared with haCGRP (Maggi et al., 1990) are very similar to those of amylin and that the highest degrees of sequence identity (80%) exist between these regions of amylin and CGRP. These results confirm the importance of the C-terminal region of CGRP and its ability to form a helix (as revealed by structure prediction methods and by the effect of TFE) in the binding to receptor. This helix-forming ability is not shown in amylin.

We are very grateful to Dr. Peter M. Bayley and Dr. Stephen Flatman for helpful discussions, and Lindsay Sawyer for some of the structural prediction analysis. We also thank the Science and Engineering Research Council for provision of the Stirling c.d. facility and Dr. S. Provencher for supplying the CONTIN program.

REFERENCES


Received 31 December 1990/11 February 1991; accepted 27 February 1991