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

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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 pK_a 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 Ca^{2+} 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 [Asn^3,Ile^{22},Ser^{25}]CGRP, [Asn^3,Asp^{14},-Phe^{15},Gly^{23}]CGRP, [Asn^3,Ser^{25}]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 pK_a 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

Abbreviations used: hαCGRP, human α-calcitonin-gene-related peptide; TFE, trifluoroethanol.

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	5	10	15	20	25	30	35
hαCGRP	Ala-Cys-Asp-Thr-Ala-Thr-Cys-Val-Thr-His-Arg-Leu-Ala-Gly-Leu-Leu-Ser-Arg-Ser-Gly-Gly-Val-Val-Lys-Asn-Asn-Phe-Val-Pro-Thr-Asn-Val-Gly-Ser-Lys-Ala-Phe-NH_2						
[Asn^3,Ile^{22},Ser^{25}]CGRP	Asn			Ile	Ser		
[Asn^3,Asp^{14},Phe^{15},Gly^{23}]CGRP	Asn		Asp Phe		Gly		
[Asn^3,Ser^{25}]CGRP	Asn				Ser		
Amylin	Lys-Cys-Asn-Thr-Ala-Thr-Cys-Ala-Thr-Gln-Arg-Leu-Ala-Asn-Phe-Leu-Val-His-Ser-Ser-Asn-Asn-Phe-Gly-Ala-Ile-Leu-Ser-Ser-Thr-Asn-Val-Gly-Ser-Asn-Thr-Tyr-NH_2						

Fig. 1. Amino acid sequences of hαCGRP, analogues of CGRP and amylin

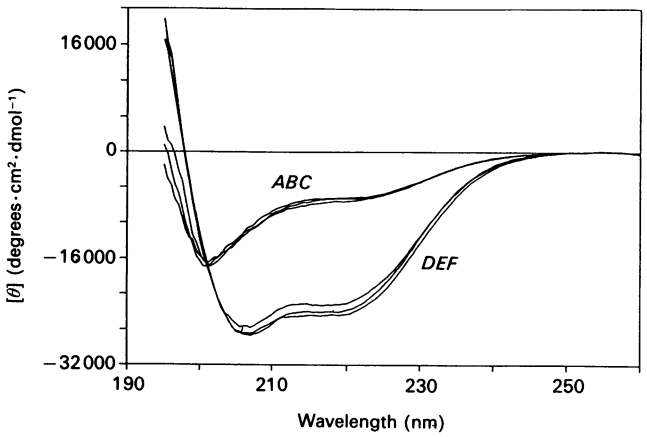

Fig. 2. Far-u.v. c.d. spectra of hαCGRP in 20 mM-sodium phosphate at pH 9 (*A*) and pH 3.5 (*B*), in water at pH 7.0 (*C*) and in 50% TFE at pH 3.5 (*D*), pH 7.0 (*E*) and pH 3.4 (*F*)

The pathlength was 1 mm, temperature was 20 °C and the protein concentration was 0.2 mg/ml.

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 hαCGRP 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 hαCGRP (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 hαCGRP: 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 hαCGRP (approx. 60%) by Manning (1989).

Near-u.v. c.d. spectra

Fig. 4 shows the near-u.v. c.d. spectra of hαCGRP in aqueous

Table 1. Structure analysis of far-u.v. c.d. spectra of hαCGRP and amylin in 20 mM-sodium phosphate

Values are given as means ± S.E.M.

	α-Helix content (%)	β-Sheet content (%)	Random coil content (%)
CGRP, 25 °C			
pH 7.0	22±3	43±5	34±5
pH 7.0 (50% TFE)	72±2	10±4	18±4
pH 7.0 (0.4% SDS)	46±2	28±3	27±3
CGRP, 50 °C			
pH 7.0	12±1	37±2	51±2
pH 7.0 (50% TFE)	56±5	0±0	44±5
pH 7.0 (0.4% SDS)	46±3	24±3	31±5
Amylin, 25 °C			
pH 7.0	13±3	16±3	71±5
pH 7.0 (50% TFE)	20±4	9±5	71±7
pH 7.0 (0.4% SDS)	62±3	34±3	4±4
Amylin, 50 °C			
pH 7.0	3±1	43±2	55±2
pH 7.0 (50% TFE)	20±2	21±3	59±3
pH 7.0 (0.4% SDS)	57±4	30±4	13±6

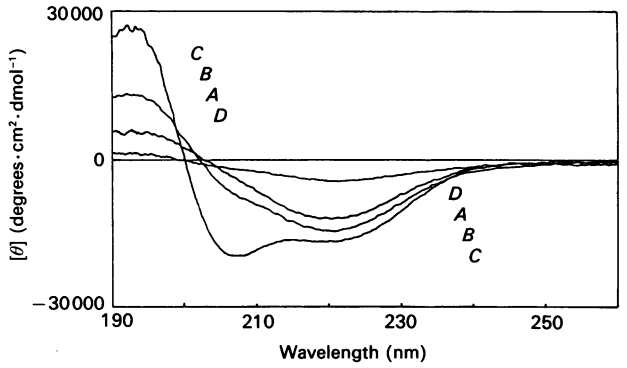

Fig. 3. Far-u.v. c.d. spectra of amylin at pH 7.0 at 25 °C in 20 mM-sodium phosphate (*A*), plus 50% TFE (*B*), or 0.4% SDS (*C*) and in 20 mM-sodium phosphate at 50 °C (*D*)

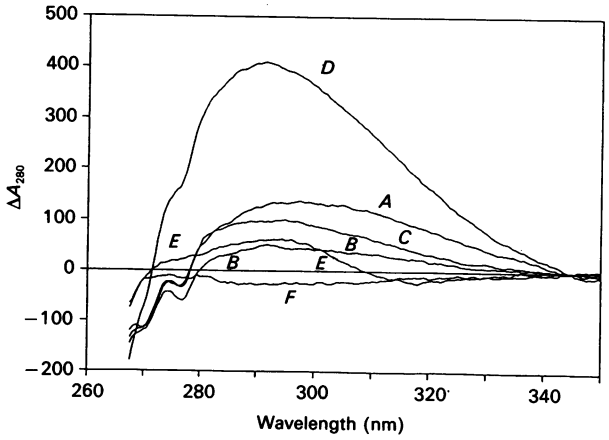

Fig. 4. Near-u.v. c.d. spectra of hαCGRP at pH 3.10 (*A*) and 7.33 (*B*) (2 mg/ml) and in 25% TFE at pH 7.76 (*C*) and 3.15 (*D*) (2 mg/ml), and after 240 min with 25 mM-dithiothreitol at pH 3.15 (*E*) and CGRP-(8–37)-peptide (*F*) (2 mg/ml)

The pathlength was 10 mm and temperature was 20 °C.

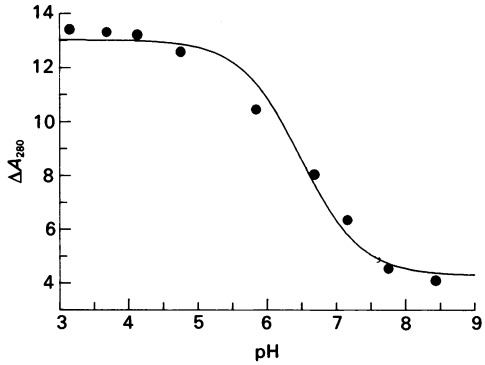

Fig. 5. pK_a of the transition of the high-pH form to the low-pH form of hαCGRP at 2 mg/ml

C.d. spectra were recorded in the near-u.v. range with a 10 mm pathlength at 20 °C. Changes in the signal at 280 nm are shown.

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 hαCGRP-(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 ΔA_{280} against pH. These data yield an apparent pK_a of 6.5 when analysed by Enzfitter software. This is consistent with the expected pK_a of histidine at residue 10 in hαCGRP, which is close to the disulphide bond at residues 2 and 7. Similar results were obtained in the absence of TFE. In contrast with hαCGRP, 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 μM-hαCGRP in aqueous solution; the helix contents at 10 μM and 1.7 mM 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 model peptides, in which a monomer/trimer equilibration occurs above 0.42 μM (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 mM), when the peptide is less structured.

Conformation of analogues of hαCGRP

The helix content of the analogues of hαCGRP (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 hαCGRP, 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

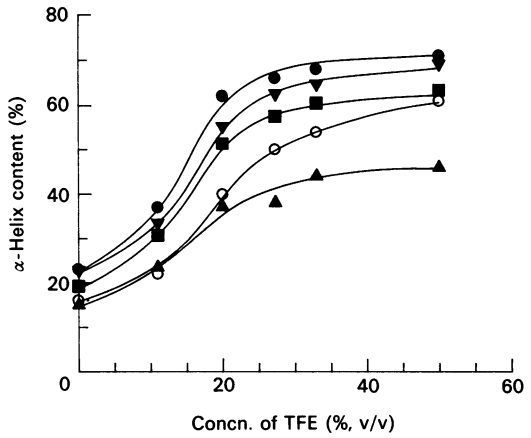

Fig. 6. Effect of TFE concentration on the α-helix content of hαCGRP and its analogues

Spectra were recorded at 20 °C with a 1 mm pathlength. ●, hαCGRP; ■, [Asn^3,Ile^{22},Ser^{25}]CGRP; ▲, [Asn^3,Asp^{14},Phe^{15},-Gly^{23}]CGRP; ▼, [Asn^3,Ser^{25}]CGRP; ○, CGRP-(8–37)-peptide.

concentrations. However, [Asn^3,Asp^{14},Phe^{15},Gly^{23}]CGRP had lower helix content (47%) than hαCGRP in 50% TFE. As this analogue had similar biological activity to hαCGRP, it suggests that formation of the high amounts of helix observed in hαCGRP 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 hαCGRP 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 hαCGRP and residues 19–23 and 29–34 in amylin. However, there is a greater tendency for β-sheet structure in hαCGRP (residues 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 hαCGRP 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 hαCGRP. Amylin was also inhibited by the CGRP antagonist CGRP-(8–37)-peptide. It is noteworthy that the biological properties of hαCGRP-(1–15)-peptide of reduced potency and duration of effects compared with hαCGRP (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.

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REFERENCES

Brain, S., Williams, T. J., Tippins, J. R., Morris, H. R. & MacIntyre, I. (1985) Nature (London) **313**, 54–56

Breeze, A. L., Harvey, T. S., Bazzo, R. & Campbell, I. D. (1991) Biochemistry **30**, 575–582

Chiba, T., Yamaguchi, T., Yamatami, T., Nakamura, A., Morishita, T., Inui, T., Fukase, M., Noda, T. & Fujita, T. (1989) Am. J. Physiol. **256**, E331–E335

Cooper, G. J. S. & Greene, H. (1989) Patent WO89/06135

Etienne, T., Girgis, S., McIntyre, I., Morris, H. R., Panico, M. & Tippins, J. R. (1984) J. Physiol. (London) **352**, 48*P*

Leighton, B. & Cooper, G. J. S. (1988) Nature (London) **335**, 632–635

Lynch, B. & Kaiser, E. T. (1988) Biochemistry **27**, 7600–7607

Maggi, C. A., Rovero, P., Ginliani, S., Evangeliata, S., Regoli, D. & Meli, A. (1990) Eur. J. Pharmacol. **179**, 217–219

Manning, M. C. (1989) Biochim. Biophys. Acta **160**, 388–392

Moe, G. R. & Kaiser, E. T. (1985) Biochemistry **24**, 1971–1976

Provencher, S. W. & Glöckner, J. (1981) Biochemistry **20**, 33–37

Tippins, J. R., Morris, H. R., Panico, M., Etienne, T., Bevis, P., Girgis, S., MacIntyre, I., Ayna, M. & Attinger, M. (1984) Neuropeptides (Edinburgh) **4**, 425–434

Sawyer, L., Fothergill-Gilmore, L. A. & Freemont, P. S. (1988) Biochem. J. **249**, 789–793

Steenberg, P. H., Hoppener, J. W. M., Zandberg, J., Lips, C. J. M. & Jansy, H. S. (1985) FEBS Lett. **183**, 403–407

Strickland, E. H. (1984) CRC Crit. Rev. Biochem. **2**, 113–175

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