A PROPOSED STRUCTURE FOR RAT CALCITONIN

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1. Introduction

Preliminary examination of rat calcitonin (RCT) isolated as described in the preceding paper indicated a great similarity with human calcitonin (HCT). Immunochemical studies by ourselves [1] and other workers [2–4] lead also to this conclusion, and moreover sequence dissimilarity with further species of calcitonin may be inferred. The amino acid composition of RCT [1] is Lys_1, His_1, Asx_3, Thr_4, Ser_2, Glx_2, Pro_2, Gly_4, Ala_2, Cys_2, Val_1, Met_1, Ile_1, Leu_3, Tyr_1, Phe_2. This differs from the composition of HCT at four points only, where the human hormone has Thr_5, Ser_1, Leu_2 and Phe_3. Thus, since only approx. 100 μg of hormone was available for sequence studies, we elected to use a comparative approach using HCT as the model.

2. Materials and methods

2.1. *Hormones*

Rat calcitonin was purified by gel filtration and column partition chromatography as described in the preceding paper. An estimated 100 μg was obtained by pooling the material remaining in both calcitonin immunoreactive peaks after preliminary characterisation. Synthetic human calcitonin CIBA-47175-Ba was a generous gift from Dr W. Rittel, Ciba-Geigy Ltd., Basel, Switzerland.

2.2. *Peptide maps*

Short peptide fragments of RCT and HCT were generated by digestion with thermolysin for 16 h at 37°C in 0.2 M pyridine acetate, pH 7.1; 200 μl for the RCT sample and 1 ml per mg for HCT. Enzyme:substrate ratios of 1:20 for RCT and 1:50 for HCT were used. The peptide fragments from each hormone were then separated on thin layers of cellulose (Merck, Darmstadt, Germany) by two-dimensional high voltage electrophoresis (pyridine–acetic acid–water, 25:1:225 by vol., pH 6.5, 50 V/cm, 30 min) and chromatography (two runs in butanol–pyridine–acetic acid–water, 42:24:4:30 by vol).

Peptide spots were located by their u.v. fluorescence after spraying with 0.05% w/v fluorescamine in 0.2% v/v pyridine in acetone [5]. Appropriate areas of the cellulose layer were then scraped off and eluted twice with 0.5 ml of the t.l.c. solvent and twice with 0.5 ml of 50%, v/v, acetic acid. The combined extracts were dried in vacuo over NaOH and P_2O_5 and then hydrolysed in 6 M HCl in the presence of 10 μg phenol at 110°C for 20 h under nitrogen in sealed tubes. Hydrolysates were analysed on a JEOL 6AH amino acid analyser (JEOL Ltd., Tokyo, Japan), equipped with a 10 mm pathlength flowcell to increase sensitivity.

2.3. *End-group and sequence determinations*

Amino terminal residue determination by dansylation and dansyl-Edman degradation were carried out as described by Hartley [6].

3. Results and discussion

Thermolytic peptide maps (fig.1) of HCT contained eleven spots. The identity of the human peptide fragments was established after elution from the plate, by inspection of their amino acid compositions and

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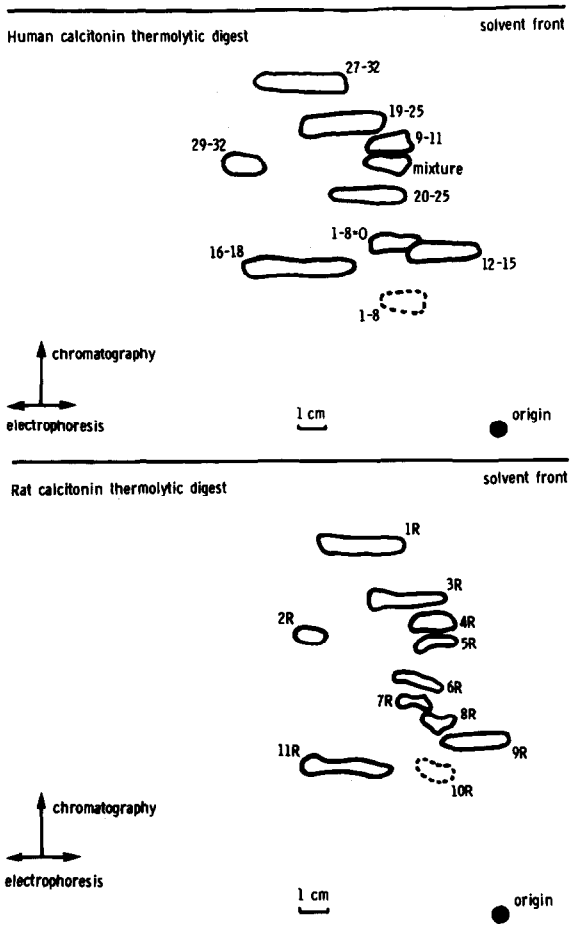

Fig.1. Peptide maps of the fragments obtained by thermolysin digestion of rat and human calcitonins. In the electrophoretic run the cathode was to the left. Peptides in the HCT digest map are labelled with the residue numbers relevant to their position in the sequence; 1–8 = 0 is the Met[8] sulphoxide derivative of the fragment sequence residues 1–8. Peptides in the rat calcitonin digest are numbered 1R–11R and their analytical data are presented in table 1.

comparison with the known sequence of human calcitonin [7]. Two of these fragments represented the same sequence, residues 1–8 of HCT, with one being the Met[8] sulphoxide (1–8 = 0) derivative and the other being the native state; one spot was a mixture of two or more unidentified fragments. All residues of HCT except Ala[26] were accounted for in these fragments, some of them more than once.

The pattern of the thermolytic fragments of RCT (fig.1) contains eleven spots, 1R–11R, which are very similar in their distribution to those obtained from HCT.

The amino acid compositions and amino terminal residue of the pure RCT fragments are listed in table 1, along with their proposed structures and position in the sequence.

These sequences were derived from the position of a rat peptide in the peptide map, its amino acid composition and its end-group and by comparison

Table 1
Thermolytic fragments of rat calcitonin

Peptide	Amino terminus	Amino acid composition	Proposed structure	(Residue numbers)
1R	0[a]	Pro Gly_2 Ala Val Ile	Ile–Gly–Val–Gly–Ala–Pro	(27–32)
2R	–[b]	Pro Gly Ala Val	Val–Gly–Ala–Pro	(29–32)
3R	0	His Thr Glx Pro Ala Phe_2	Phe–His–$^{Ala}/_{Thr}$–Phe–Pro–Gln–$^{Thr}/_{Ala}$	(19–25)
4R	Leu	Thr Gly Leu	Leu–Gly–Thr	(9–11)
5R	Leu	Mixture	–	
6R	–	Mixture	–	
7R	–	Mixture	–	
8R	–	Mixture	–	
9R	Tyr	Asx Thr Glx Tyr	Tyr–Thr–Gln–Asp	(12–15)
10R	–	Mixture	–	
11R	Leu	Lys Asx Leu	Leu–Asn–Lys	(16–18)

[a] 0 = no end group detected.
[b] – = dansylation not done.

with data for the corresponding human peptide. Amide assignments were made by analogy with residues in HCT since in each case fragments from RCT containing Asx and Glx had the same electrophoretic mobility as the corresponding peptide from HCT. The C-terminal fragments of RCT also had mobilities consistent with C-terminal proline being amidated.

Low yields were obtained for amino terminal residues on amino acid analysis (except tyrosine in peptide 9R) no doubt due to blocking by fluorescamine. This confirmed results of dansylation, where obtained, and suggested Ile and Val as N-termini for sequences 27–32 and 29–32 respectively where no dansyl end group could be obtained.

On this basis the rat calcitonin peptides 1R, 2R, 4R and 9R are identical to the peptides of sequence residues 27–32, 29–32, 9–11 and 12–15 respectively in human calcitonin.

Rat peptide 11R corresponds in the peptide map with the human peptide sequence residues 16–18 but the amino acid composition indicates the presence of leucine in place of the phenylalanine found in HCT. Dansylation shows this to be amino terminal and so the sequence is most probably Leu–Asn–Lys.

The information available does not allow a definite assignment of the exchange seen in peptide 3R which has an overall resemblance to the sequence residues 19–25 in human calcitonin. Here one of the two threonines (21 and 25) in HCT appears to have been replaced by alanine in RCT but there is no additional evidence to decide which.

The peptides isolated in pure form account for residues 9–25 and 27–32 of rat calcitonin and leave, by subtraction from the total amino acid composition, Cys_2, Gly, Asx, Leu, Thr, Ser_2, Met to fill sequence positions 1–8 and 26.

Dansylation of the performic acid oxidised native RCT gave cysteic acid at sequence position one [1]. Dansyl-Edman degradation gave glycine for residue two.

Mild oxidation with hydrogen peroxide to convert methionine to its sulphoxide destroyed the biological activity of RCT. This is characteristic of the two calcitonins (human and salmon III) which have methionine at position 8 [8,9]. Other species (porcine, ovine and bovine) with methionine at position 25 retain biological activity after mild oxidation [10]. It

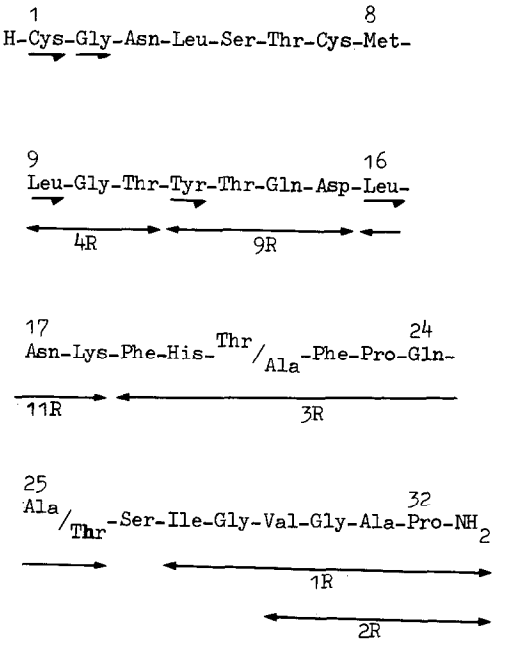

Fig.2. Proposed sequence for rat calcitonin. Numbered double-headed arrows indicate the peptides isolated by t.l.c. and listed in table 1. (⟶) Residues determined by dansylation. Positions 21 and 25 are occupied by alanine and threonine but the correct assignment could not be made. Residues numbers 1, 3, 4, 5, 6, 7, 9, 28 and 32 are those normally conserved in calcitonins.

is likely, therefore, that RCT has methionine at position 8.

If serine is then placed at position 26 the remaining amino acids may be placed in positions 3–7 in agreement with those normally found in this conserved region within calcitonins.

We therefore propose the sequence shown in fig.2 for rat calcitonin. This sequence shows extensive homology with HCT which has Phe-16, Thr-21, Thr-25 and Ala-26 in place of those shown.

RCT is the first calcitonin to show such a large degree of homology with HCT. The amino acid exchanges found are in the C-terminal half of the molecule where antigenicity resides [11,12]. They are too few to destroy binding to antibodies but clearly explain the reduced binding energy observed in the radioimmunoassay based on HCT [1].

It is interesting to note that RCT and HCT have closely similar structures and were both isolated from

thyroid medullary carcinoma. Both species differ markedly from all other known sequences which themselves fall into two distinct groups, typified by salmon and porcine calcitonins, and were isolated from normal tissue.

However other work on rat calcitonin has employed material from normal thyroid tissue and indicates probable identity between 'normal' and 'tumour' rat calcitonins. In particular the provisional amino acid composition for 'normal' RCT published by Burford et al. [2] is essentially the same as that of our 'tumour' RCT. And, in addition, extracts of normal rat thyroid and extracts of thyroid medullary carcinoma both cross-react in radioimmunoassays for human calcitonin [1–4].

We therefore feel that the structure in fig.2 represents normal rat calcitonin.

Acknowledgement

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