STRUCTURE OF THE VASOACTIVE INTESTINAL OCTACOSAPEPTIDE FROM CHICKEN INTESTINE. THE AMINO ACID SEQUENCE

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1. Introduction

In a previous paper [1] we reported that the chicken vasoactive intestinal octacosapeptide, like the porcine vasoactive intestinal octacosapeptide, glucagon and secretin has an N-terminal histidyl-seryl structure. A residue of threonine amide constitutes its C-terminus as compared to asparagine amide in the porcine peptide. The complete amino acid sequence of the polypeptide has now been elucidated, and the present paper gives the evidence for the proposed sequence.

2. Materials and methods

Chicken vasoactive intestinal peptide was prepared as described earlier [1]. The sources and qualities of the other materials used have also been described previously, as have the methods used for paper electrophoresis and paper chromatography of peptides, for the determination of their N-terminal amino acids, for their enzymatic fragmentation, total hydrolysis, enzymatic or with acid, and for the determination of the amino acid compositions of the hydrolysates [2].

3. Results

3.1. *Cleavage of the octacosapeptide with kallikrein and separation of the fragments*

4 mg of the octacosapeptide [1] was degraded with kallikrein [2]. Electrophoresis of an aliquot at pH 6.4 showed that it had been split into one neutral and one basic fragment. The bulk of the degraded material was dissolved to a 1% solution in 0.02 M NH_4HCO_3 adjusted to pH 6.5 with CO_2 and followed by this buffer, passed through a column (0.6 × 15 cm) of carboxymethyl-cellulose, CMC, (Whatman CM-22) which had been equilibrated with the same buffer. A fraction of 5 ml was collected whereupon the buffer was changed to 0.04 M NH_4HCO_3 and a second fraction of 5 ml collected. The buffer was changed to 0.08 M, 0.16 M, and 0.4 M NH_4HCO_3 and one fraction of 5 ml collected from each. The fractions were lyophilized. The first fraction contained the neutral and the fourth fraction the basic fragment. See fig.1. The neutral fragment, which gave a positive Pauly reaction was denominated CV-KN; it weighed 1.4 mg. The basic fragment gave a negative Pauly reaction and was denominated CV-KC; it weighed 1.1 mg.

3.2. *Amino acid sequence of CV-KN*

3.2.1. Fragmentation of CV-KN with trypsin.

On degradation of 1.4 mg CV-KN with trypsin two fragments were again formed: one with cathodic electrophoretic mobility at pH 6.4 and one with anodic mobility and a positive Pauly reaction (fig.2). The peptide mixture was applied to a CMC column (0.6 × 11.5 cm) in 0.02 M NH_4HCO_3. Two fractions of 4 ml were collected whereupon the eluant was changed to 0.2 M NH_4HCO_3 and two more fractions of 4 ml each collected. The first fraction contained the Pauly positive fragment which was denominated CV-KN-TrN. It weighed about one mg. The third fraction contained the Pauly negative one which was denominated CV-KN-TrC. Qualitative amino acid analysis of hydrolyzed samples of it contained only phenylalanine and arginine.

Fig.1. Paper electrophoresis of kallikrein degradation products of the chicken vasoactive intestinal octacosapeptide. (a) 60 μg chicken vasoactive-octacosapeptide. (b) Kallikrein degradation mixture from 60 μg chicken vasoactive octacosapeptide. (c and d) Separation of the kallikrein degradation mixture on a column of CMC, according to section 3.1. of text: (c) 30 μg lyophilized material from the first fraction of the CMC-column. It was Pauly positive and denominated CV-KN. (d) 30 μg lyophilized material from the fourth fraction of the CMC-column. It was denominated CV-KC. Electrophoresis was performed for 90 min at 50 V/cm in pyridine–acetic acid–water (300:11. 5:2700, by vol) at pH 6.4 using Whatman 3MM paper. Staining with the cadmium-ninhydrin reagent of Barrolier et al. [3].

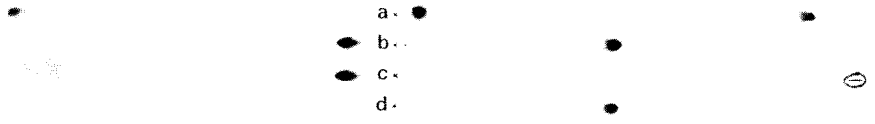

Fig.2. Paper electrophoresis of fragments obtained on degrading the N-terminal tetradecapeptide, CV-KN, of the chicken vasoactive intestinal octacosapeptide with trypsin. (a) Reference substances: Aspartic acid, taurine and lysine, each 20 nmol. (b) Tryptic degradation mixture from 50 μg CV-KN. (c and d) Separation of the tryptic degradation mixture on a column of CMC, according to section 3.2. of text: (c) Lyophilized material from the first fraction of the CMC-column corresponding to 50 μg of CV-KN. It was Pauly positive and denominated CV-KN-TrN. (d) Lyophilized material from fraction three of the CMC-column corresponding to 50 μg of CV-KN. It was denominated CV-KN-TrC. Electrophoresis conditions as in fig.1.

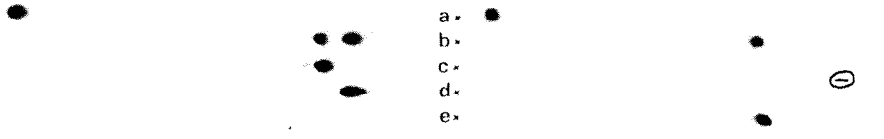

Fig.3. Paper electrophoresis of the fragments obtained on degrading the N-terminal dodecapeptide, CV-KN-TrN, of the chicken vasoactive intestinal octacosapeptide with chymotrypsin. (a) Reference substances: aspartic acid and taurine, each 20 nmol. (b) Chymotryptic degradation mixture of 50 μg CV-KN-TrN. (c) Five per cent aliquot of lyophilized fractions 4–7 from separation of chymotryptic degradation mixture of 1 mg of CV-KN-TrN on SE-Sephadex column (0.6 × 23 cm) with an ammonia/acetic acid buffer of pH 4.8 [2]. It gave a colour in the Pauly reaction like that given by tyrosine. It was Sakaguchi negative and denominated CV-KN-TrN-Chti. (d) Five per cent aliquot of lyophilized fractions 28–37. It gave a colour in the Pauly reaction like that given by histidine. It was Sakaguchi negative and denominated CV-KN-TrN-ChtN. (e) Five per cent aliquot of lyophilized fractions 49–51. Buffer changed at fraction 44 to 0.2 M NH_4HCO_3:0.2 M NH_3 (1:1) It was Sakaguchi positive and Pauly negative. It was denominated CV-KN-TrN-ChtC. Electrophoresis conditions as in fig.1.

3.2.2. Fragmentation of CV-KN-TrN with chymotrypsin.

CV-KN-TrN was degraded with chymotrypsin into three fragments: One with cathodic mobility at pH 6.4 and two with anodic mobilities, fig.3. The fragment with cathodic mobility gave a positive Sakaguchi and a negative Pauly reaction. With the cadmium-ninhydrin reagent it gave an orange–red colour. It was denominated CV-KN-TrN-ChtC. The fragment with the lower anodic mobility gave a colour in the Pauly reaction like that given by histidine. It was Sakaguchi negative and denominated CV-KN-TrN-ChtN. The fragment with higher anodic mobility gave a colour in the Pauly reaction like that given by tyrosine and was Sakaguchi negative. It gave a brilliant yellow colour with the ninhydrin-cadmium reagent and was denominated CV-KN-TrN-Chti. The three fragments were separated on a column (0.6 × 23 cm) of SE-Sephadex similarly to the separation of the three chymotryptic fragments of the N-terminal tryptic dodecapeptides of porcine vasoactive intestinal peptide [2] and secretin [3], fig.3.

3.2.3. Evidence for the sequence of CV-KN

Determination of N-terminal amino acid residues, qualitative amino acid analysis of acid or enzymatic (aminopeptidase M) hydrolyzates and the specificity of the fragmentation agents used gave, when correlated with the known quantitative amino acid composition of CV-KN, the following information about these subfragments of the tetradecapeptide:

CV-KN-TrN-ChtN was His(Ala,Asp,Ser,Val)Phe.
CV-KN-TrN-Chti was Thr(Asn,Asp)Tyr.
CV-KN-TrN-ChtC was Ser–Arg.
CV-KN-TrC was Phe–Arg.

This suggested that CV-KN-TrN-ChtN and CV-KN-TrN-Chti might be identical with the corresponding porcine peptides [2]. They were therefore subjected to amino acid sequence determination exactly as described for the latter [2,4] and the suspected identities were indeed confirmed. These results show that the 14 first amino acids of the chicken octacosapeptide are in the sequence: His–Ser–Asp–Ala–Val–Phe–Thr–Asp–Asn–Tyr–Ser–Arg–Phe–Arg.

3.3. *Amino acid sequence of CV-KC*

3.3.1. Fragmentation with cyanogen bromide

CV-KC, 1.1 mg, was treated with cyanogen bromide and passed through a DEAE-Sephadex column (0.6 × 105 cm) in 0.2 M acetic acid [2]. The degraded chloride-free lyophilized material was dissolved in 1 ml M acetic acid and chromatographed in this solvent on a column of Sephadex G-25 (0.6 × 105 cm). Flow rate, 0.5 ml/4 min, 60 fractions were collected. All fractions were lyophilized and then reconstituted in 50 μl of water. Aliquots of 1 μl from each fraction were applied to a strip of Whatman 42 filter paper and the strip was, after drying, drawn through the cadmium-ninhydrin solution. The material from fractions 23–30 and 32–35 gave a positive ninhydrin reaction. An acid hydrolyzate of an aliquot of the combined fractions 23–30 showed that the material contained alanine, aspartic acid, leucine, lysine, threonine, tyrosine, serine, and valine. After lyophilization the material was denominated CV-KC-CNBr-C. A similar hydrolyzate of fraction 32–35 contained homoserine, glutamic acid and lysine. It was denominated CV-KC-CNBr-N. In this tripeptide the lysine was shown to be N-terminal, and, electrophoretic evidence indicated that glutamine rather than glutamic acid was present in the intact peptide.

3.3.2. Degradation of CV-KC-CNBrC with chymotrypsin.

When CV-KC-CNBrC was degraded with chymotrypsin three fragments, one neutral and two basic, were formed as shown by paper electrophoresis at pH 6.4 (fig.4.) The basic fragment with highest electrophoretic mobility gave a canary yellow colour with the cadmium-ninhydrin reagent similar to that given by peptides with N-terminal threonine. Its electrophoretic mobility was identical with that of threonine amide, shown earlier to be C-terminal in chicken VIP [1]. The other two fragments gave the usual reddish colour. The separation of the three fragments on a CMC column (0.6 × 25 cm) with 0.02 M NH_4HCO_3 adjusted to pH 6.5 with CO_2, and then 0.2 M NH_4HCO_3 followed closely the similar separation of the corresponding fragments from the porcine material [2]. 55 fractions of 1 ml each were collected. The change of buffer was made after the 40th fraction. On paper electrophoresis of aliquots it was found that the material from fractions 7–11 constituted the neutral chymotryptic fragment of CV-KC-CNBrC. It was found to have N-terminal leucine and was denominated CV-KC-CNBrC-Chti. The material from fractions 19–23 was the basic

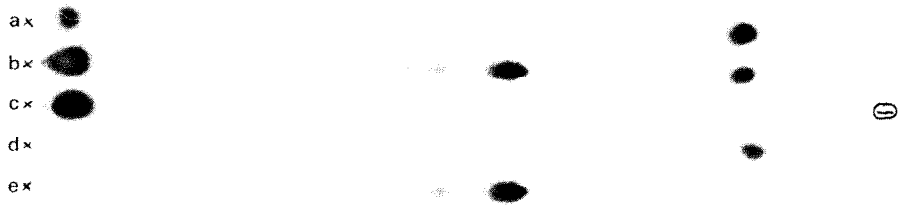

Fig.4. Paper electrophoresis of the fragments obtained on degrading the C-terminal undecapeptide, CV-KC-CNBrC, of the chicken vasoactive intestinal octacosapeptide with chymotrypsin. (a) Reference substances: threonine amide and taurine, each 20 nmol. (b) Chymotryptic degradation mixture of 50 μg CV-KC-CNBrC. (c-e) Separation of the chymotryptic degradation mixture on a column of CMC, according to section 3.3. of text: (c) Aliquot of lyophilized fractions 7–11 corresponding to 50 μg of CV-KC-CNBrC, from the CMC-column. It was denominated CV-KC-CNBrC-Chti. (d) Aliquot of lyophilized fractions 19–23 corresponding to 50 μg of CV-KC-CNBrC, from the CMC-column. It gave a canary yellow colour with the cadmium-ninhydrin reagent like that given by Thr-NH_2. It was indistinguishable in tone from that given by the reference substance. It was denominated CV-KC-CNBrC-ChtC. (e) Aliquot of fractions 47–50 corresponding to 50 μg of CV-KC-CNBrC, (from the CMC-column). It was denominated CV-KC-CNBrC-ChtN. Electrophoresis conditions as in fig.1.

fragment with highest electrophoretic mobility and the yellow colour with the ninhydrin reagent, and was denominated CV-KC-CNBrC-ChtC. The material from fractions 47–50 was the basic fragment with lower electrophoretic mobility and 'ordinary' reddish colour with the ninhydrin reagent. It was found like CV-KC-CNBrC itself to have N-terminal alanine and was denominated CV-KC-CNBrC-ChtN.

3.4. *The amino acid compositions and sequences of the three chymotryptic fragments*

Paper chromatography of an acid hydrolyzate showed that CV-KC-CNBrC-ChtN contained the same amino acids as the corresponding peptide from the porcine octacosapeptide, suggesting that their sequences also might be identical. This was shown to be so by subjecting the chicken peptide to the same treatment as had been used to elucidate the sequence of the porcine peptide i.e. showing that its N-terminal amino acid was alanine and splitting it by trypsin into the two fragments which were identified as Ala–Val–Lys and Lys–Tyr.

CV-KC-CNBrC-Chti was found to have N-terminal leucine, like the corresponding porcine peptide, but it differed from the latter by containing valine and no isoleucine. It was subjected to the same treatment that had been used for elucidating the sequence of the corresponding porcine peptides. Thermolysin split it into two fragments which were isolated by paper chromatography in the Waley-Watson system, and identified as Leu (Asn,Ser) and Val–Leu. Proteinase K split it into two main fragments identified as, Leu–Asn and Ser–Val–Leu, and besides, to a smaller extent, into the same fragments as given by thermolysin. The combined results with these two enzymes established the sequence of the neutral chymotryptic pentapeptide as Leu–Asn–Ser–Val–Leu. An acid hydrolyzate of the basic fragment from fractions 19–23 contained threonine only. It has already earlier [1] been shown that the C-terminus of the chicken vasoactive octacosapeptide is threonine amide.

These results gave the following information about the subfractions of the C-terminal tetradecapeptide CV-KC:

CV-KC-CNBrN was Lys–Gln–Homoserine.
CV-KC-CNBrC-ChtN was Ala–Val–Lys–Lys–Tyr.
CV-KC-CNBrC-Chti was Leu–Asn–Ser–Val–Leu.
CV-KC-CNBrC-ChtC was ThrNH_2.

The sequence of the chicken vasoactive intestinal octacosapeptide is consequently His–Ser–Asp–Ala–Val–Phe–Thr–Asp–Asn–Tyr–Ser–Arg–Phe–Arg–Lys–Gln–Met–Ala–Val–Lys–Lys–Tyr–Leu–Asn–Ser–Val–Leu–ThrNH_2.

This differs from the corresponding porcine sequence by the replacement of Thr-11 by Ser-11, of Leu-13 by Phe-13, of Ile-26 by Val-26 and by the C-terminal residue of asparagine amide by a residue of threonine amide.

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