Volume 255, number 1, 10–14 FEBS 07565 September 1989

Amino acid sequence of the crustacean hyperglycemic hormone (CHH) from the shore crab, *Carcinus maenas*

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Received 28 June 1989

Crustacean hyperglycemic hormone (CHH) was isolated from sinus glands of *Carcinus maenas*, and its primary structure was determined by manual microsequencing, using the DABITC-PITC double-coupling method. The neurohormone consists of 72 amino acid residues (8524 Da). Three disulfide bridges are present and both the N- and C-terminus are blocked. CHH does not show significant sequence homology to any known peptide hormone or protein.

Amino acid sequence; Crustacean hyperglycemic hormone; Neuropeptide; (*Carcinus maenas*)

1. INTRODUCTION

The existence of a 'diabetogenic factor' in the crustacean eyestalk was demonstrated more than 40 years ago [1]. Subsequent studies on the physiology and biochemistry of this 'crustacean hyperglycemic hormone (CHH)', as it became known, have been summarized in recent reviews [2,3]. In addition to its well-documented role as a blood glucose-regulating hormone, CHH has been found to be a potent secretagogue, releasing digestive enzymes from the hepatopancreas [3]. Thus, it may be a metabolic hormone with multiple functions.

Antisera to CHH, employed in immunocytochemical studies, have revealed a distinct, classical neurosecretory pathway in the eyestalk, consisting of perikarya in the so called X-organ in the medulla terminalis and axon endings in the neurohemal sinus gland (SG) [2,4]. By radioimmunoassay [5], resting hemolymph levels and elevated levels under physiological stress could be measured [6], confirming a hormonal role of CHH in vivo.

Due to the abundance of CHH-axon terminals, the SG is an excellent, enriched source for isolation of the hormone. Isolation of CHH by a simple two-step HPLC procedure, from *Carcinus maenas* and some other species, and amino acid composition data, have previously been reported from this laboratory [2,7,8].

Here we report the complete amino acid sequence of CHH from the shore crab, *C. maenas*.

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Abbreviations: DABITC, 4-*N,N*-dimethylaminoazobenzene 4′-isothiocyanate; DABTH, 4-*N,N*-dimethylaminoazobenzene thiohydantoin; PITC, phenylisothiocyanate; HPLC, high-performance liquid chromatography; TFA, trifluoroacetic acid.

2. MATERIALS AND METHODS

2.1. *Isolation of CHH*

Dissection and extraction of sinus glands and purification of CHH by HPLC have been described [7,8]. HPLC yields two CHH peaks, a minor pre-peak (approx. 10% of total) and a slightly later eluting main peak. Both forms do not differ in hyperglycemic activity, and no significant differences in amino acid composition could be demonstrated. This paper describes the sequencing of the main peak material.

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00145793/89/$3.50

2.2. *Derivatization of disulfide bonds*

2.2.1. Oxidation

Performic acid oxidation was performed according to [9].

2.2.2. Reduction and alkylation

Samples were dissolved in 100 mM Tris-HCl (pH 8.3) containing 6 M guanidine HCl and 2 mM EDTA, and incubated for 1 h at 37°C. Reduction with dithiothreitol (DTT) and alkylation with iodoacetic acid were performed as in [10]. Acetic acid (HOAc) was added to a final concentration of 50%. S-CM-CHH was separated on a Sephadex G-25 column (0.6 × 13 cm), with 50% HOAc as eluant, and lyophilized.

Pyridylethylation: Reduction and reaction with 4-vinylpyridine were performed according to [11], but with 100 mM Tris-HCl (pH 8.3) as buffer. *S*-Pyridylethyl-CHH (S-PE-CHH) was isolated by HPLC as described in section 2.4 (conditions b).

2.3. *Enzymatic digestion*

2.3.1. Trypsin (TPCK-treated, Worthington)

Oxidized and S-CM-CHH: Samples were incubated in 1% NH_4HCO_3 buffer (pH 8.8) for 4 h at 37°C at an enzyme/substrate ratio of 1:30 (w/w). Native CHH and endoprotease Asp-N-generated fragments of native CHH: Incubations were carried out in 100 mM Tris-HCl (pH 8.3) containing 5% acetonitrile (CH_3CN) for 15 h at 37°C; the enzyme/substrate ratio was 1:20.

2.3.2. Endoprotease Glu-C (Boehringer sequencing grade)

Digestion was carried out in 100 mM NH_4HCO_3 buffer (pH 7.8), containing 2 mM EDTA, for 8 h at 37°C; enzyme/substrate ratio, 1:30.

2.3.3. Endoprotease Asp-N (Boehringer sequencing grade)

Native, oxidized and S-PE-CHH was digested in 50 mM Tris-HCl (pH 8.0) for 18 h at 37°C. In the case of S-PE-CHH, 10% CH_3CN was added as the material was poorly soluble at pH 8.0; enzyme/substrate ratio, 1:100.

2.3.4. Carboxypeptidase P (Boehringer sequencing grade)

About 8 nmol C-terminal tryptic fragment (T2) were treated with 400 ng enzyme in 50 μl of 50 mM sodium phosphate buffer (pH 6.5) for 15 h at 25°C.

2.3.5. Pyroglutamate-aminopeptidase (Boehringer)

The N-terminal tetrapeptide (G2) was cleaved in 100 mM Tris-HCl (pH 7.5), containing 4 mM DTT and 4 mM EDTA, for 60 min at 30°C. Of the enzyme, 2/3 were added initially and 1/3 after 15 min (final enzyme/substrate ratio, 40:1, w/w). Reaction was stopped by dilution with 4 N HOAc.

2.4. *Separation of fragments*

This was achieved by HPLC on a μBondapak phenyl column (0.39 × 30 cm, Millipore-Waters) with linear gradient elution at a flow rate of 0.9 ml/min and monitoring at 210 mm. Solvent A, 0.11% TFA; solvent B, 0.10% TFA/60% CH_3CN. Two protocols were used: (a) 10–80% B in 90 min, (b) 10–80% B in 60 min.

2.5. *Amino acid analysis*

A Biotronic LC 5000 analyzer with ninhydrin postcolumn reaction was used to determine amino acid compositions and quantities of peptides after hydrolysis with constant-boiling HCl in vacuo for 1 h at 150°C [11].

2.6. *Microsequencing*

Sequencing was carried out manually by the DABITC-PITC double-coupling method [12]. DABTH amino acids were separated and identified by HPLC on a Nucleosil C18 column (3.9 × 30 cm, particle size 5 μm, Macherey and Nagel). Solvent A, 35 mM sodium acetate buffer (pH 5.0); solvent B, CH_3CN. A linear gradient from 30 to 70% B in 60 min at a flow rate of 0.5 ml/min was used, with monitoring at 430 nm.

2.7. *FAB-mass spectrometry*

FAB-MS was performed on a ZAB HF instrument (VG Analytical, UK). The peptide (1 μg) in aqueous solution was applied to the target which was coated with thioglycerol as matrix. The target was bombarded with xenon atoms at a kinetic energy equivalent to 8.5–9.5 kV. A single-scan spectrum was run in the positive ion mode.

3. RESULTS

A complete set of tryptic fragments of CHH was obtained by HPLC (fig.1). Manual sequencing of these fragments (except for the blocked N-terminal T4) and of overlapping fragments from endoprotease Glu-C and Asp-N digestions yielded the sequence from position 5–71. The retention times of the overlapping fragments are given in table 1. The amino acid composition of all fragments was determined before sequencing. The sequencing strategy is shown in fig.2. There was no clear indication of microheterogeneity at any step during the sequencing.

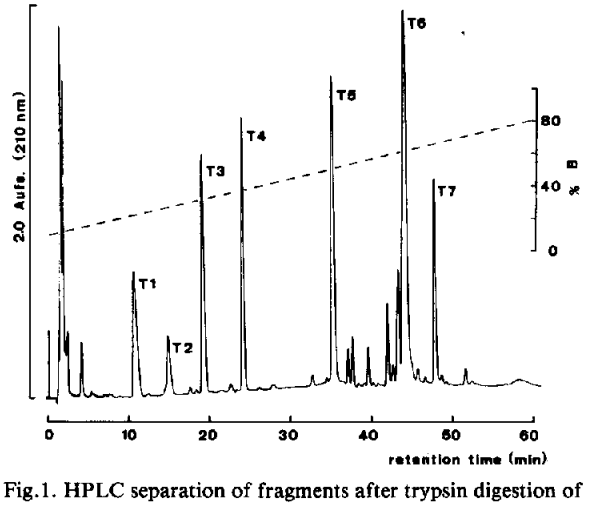

Fig.1. HPLC separation of fragments after trypsin digestion of 200 μg (23 nmol) S-CM-CHH. For details see section 2.

Table 1

HPLC retention times of endoprotease Asp-N and Glu-C digestion-derived fragments[a]

$A1_n$	30% B	conditions b
$A5_n$	59% B	
$A9_{ox}$	33% B	
$A19_{ox}$	48% B	
$A9_p$	55% B	
G1	25% B	conditions a
G2	29% B	
G5	38% B	
G6	44% B	

[a] For abbreviations see legend to fig.2

The blocked, carboxymethylated N-terminal octapeptide (T4) was analyzed by FAB-MS. No sequence data were obtained, but the molecular ion showed a mass deficit of 18, suggesting pGlu as the first residue. This was supported by the finding that T4 could be cleaved by Glu-C-protease to yield the N-terminal tetrapeptide G2, which proved susceptible to pyroglutamate-aminopeptidase digestion.

C-terminal analysis: After incubation of T2 (positions 68–72) with carboxypeptidase P, only Met, known to be in position 71 from the sequencing of $A9_{ox}$ (fig.2), was found by amino acid analysis. HPLC of the enzyme digest under conditions b yielded uncleaved T2 and a tetrapeptide with only one Val. In the void volume, free Met could be detected by amino acid analysis. Hydrolysis of the void material gave, in addition to Met, the residues Lys, Val and Glx which indicated the existence of the uncleaved N-terminal tripeptide of T2. However, Val appeared in much higher than equimolar quantities. This could only be ascribed to deamidation, by acid hydrolysis, of Val-NH_2 which was unexpectedly cleaved from T2 by carboxypeptidase P.

Assignment of disulfide bridges: Trypsin cleavage of native CHH yielded a fragment eluting at 58% B on HPLC (conditions b). Its amino acid

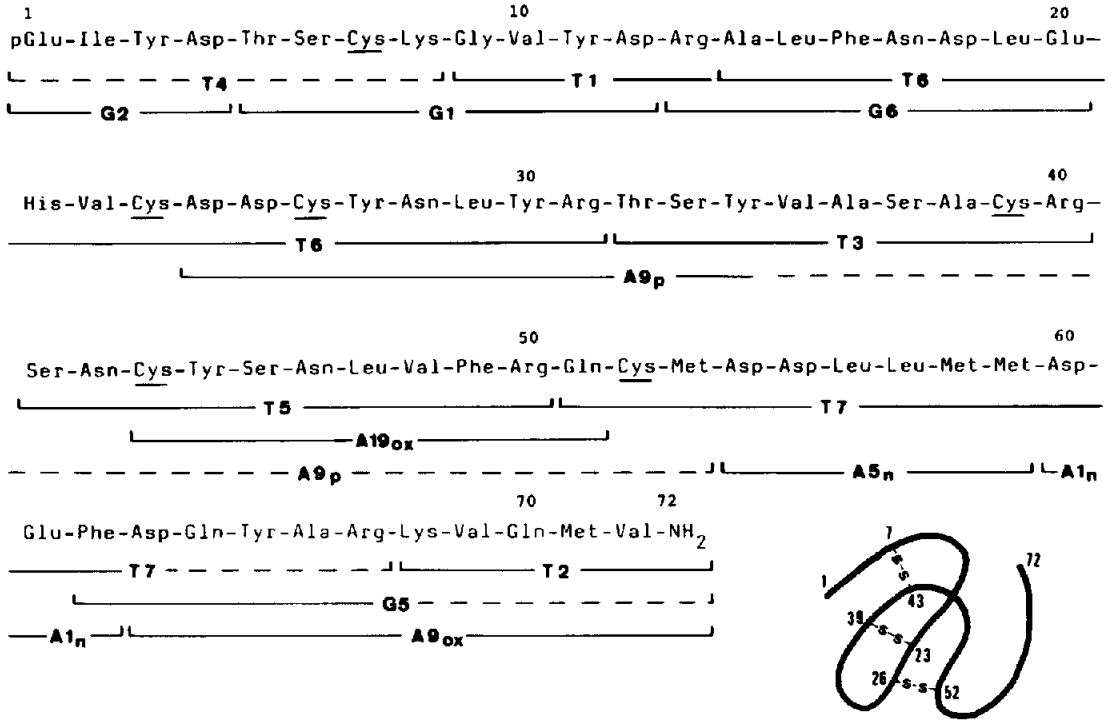

Fig.2. Amino acid sequence of CHH from *Carcinus maenas* and positions of disulfide bonds. T, tryptic fragments; G, fragments from endoprotease Glu-C digestions of S-CM-CHH; A, fragments from endoprotease Asp-N digestions of native (n), pyridylethylated (p) and oxidized (ox) CHH. Note additional cleavage sites N-terminally of cysteic acid residues. Dashed lines: residues not sequenced but consistent with amino acid composition. Numbering of fragments according to their positions in HPLC chromatograms. Cys residues underlined.

composition was only consistent with a linkage of T4 and T5, i.e. by a bridge between positions 7 and 43. In an endoprotease Asp-N-digest of native CHH, a fragment eluting at 60% B (conditions b) appeared whose amino acid composition corresponded to positions 4–11 plus 18–53. It was further subjected to trypsin digestion and separated under the above conditions. One product, eluting at 42% B, had a composition accounting for positions 18–23 plus 32–40, permitting assignment of the disulfide bridge 23–39. Another minor fragment eluting at 52% B had a composition consistent with positions 24–31 plus 51–53, yielded the third disulfide bridge 26–52. Thus, the positions of all three disulfide bridges could be determined unambiguously.

4. DISCUSSION

The determination of the amino acid sequence of CHH from *C. maenas* showed the hormone to be a larger molecule than expected from earlier gel-filtration and amino acid composition data [2,13]. In accordance with the results of others on CHHs from different species by SDS electrophoresis [14], these data suggested a minimal molecular mass of between 6 and 7 kDa, in contrast to the value of 8524 Da, as calculated here from the complete sequence. Also, two disulfide bridges were assumed instead of the three that are actually present. An interesting feature of the sequence is the dibasic sequence Arg-Lys (positions 67–68) near the C-terminus, a potential cleavage site which is apparently not used. It is currently under investigation as to whether the truncated variant 1–65 exists in the SG, and whether the N-terminal peptide Val-Glu-Met-Val-NH_2 has a specific biological activity of its own.

A search of the protein and DNA-databank (GenBank at The Los Alamos National Laboratory) did not reveal any significant sequence homology to a known peptide hormone, neuropeptide or protein. However, a scan of the databank with the cysteine core of CHH, allowing variable amino acids in between, revealed that the central core of four Cys residues (positions 23, 26, 39, 43) is identically present in the eclosion hormone of *Manduca sexta* [19]. Otherwise, there is not a single other identical amino acid in this region. The significance, if any, of this finding remains obscure at present.

CHH appears to be a member of a novel peptide family. There is evidence of the existence of several important related hormones in the crustacean X-organ-SG system. A molecular mass in the range 6–8 kDa seems to be a common feature. In addition to our observation of two peaks with CHH activity in *Carcinus*, more than one variant was also demonstrated in other species [14]. Peptide hormones with gonad- and vitellogenesis-inhibiting activity [15] and the so-called moult inhibiting hormone (MIH) may also belong to this family [8,16,18]. It is interesting that a value of 8.5 kDa, very similar to that of *Carcinus* CHH, has been reported for the MIH of the lobster [16]. We have isolated the MIH of *Carcinus* and found it to be similar in size and amino acid composition to CHH [8]. The fact that CHH has some MIH activity indicates structural similarity. A certain degree of immunological cross-reactivity of several lobster peptides is also suggestive in this respect [15,17].

In conclusion, there appear to be several peptides present in one species, whose structural similarity is reflected in a certain degree of overlap in biological activity. In view of the active research currently being performed in this field, we can expect to see more of these peptides completely sequenced in the near future. These data will provide a basis for understanding the evolution of the peptides, both intra- and interspecifically, and the emergence of different physiological functions.

Acknowledgments: This study was supported by successive grants from the Deutsche Forschungsgemeinschaft (Ke 206/7). We thank Dr S.G. Webster, Bangor, for his generous help in procuring sinus glands and purified CHH, and Dr J.W. Slootstra, Free University Amsterdam, for performing the GenBank search.

REFERENCES

[1] Abramowitz, A.A., Hisaw, F.L. and Papandrea, D.N. (1944) Biol. Bull. 86, 1–5.
[2] Keller, R., Jaros, P. and Kegel, G. (1985) Am. Zool. 25, 207–221.
[3] Keller, R. and Sedlmeier, D. (1988) in: Invertebrate Endocrinology (Laufer, H. and Downer, R.G.H. eds) vol. 2, pp. 315–326, A.R. Liss, New York.
[4] Dircksen, H., Webster, S.G. and Keller, R. (1988) Cell Tissue Res. 251, 3–12.

[5] Keller, R. (1988) in: Immunological Techniques: Arthropods (Gilbert, L.I. and Miller, T.A. eds) Springer Ser. Exp. Entomol., pp. 253–272, Springer, Heidelberg.
[6] Orth, H.P. (1989) PhD Dissertation, University of Bonn.
[7] Keller, R. and Kegel, G. (1984) in: Biosynthesis, Metabolism and Mode of Action of Invertebrate Hormones (Hoffman, J.A. and Porchet, J.A. eds) pp. 145–154, Springer, Heidelberg.
[8] Webster, S.G. and Keller, R. (1986) J. Comp. Physiol. B. 156, 617–624.
[9] Hirs, C.H.W. (1967) Methods Enzymol. 11, 197–199.
[10] Allen, G. (1981) Sequencing of Proteins and Peptides, Elsevier, Amsterdam.
[11] Tarr, G.E. (1986) in: Methods of Protein Microcharacterization (Shively, J.E. ed.) pp. 155–194, Humana, Clifton.
[12] Chang, J.-Y. (1981) Biochem. J. 199, 557–564.
[13] Kleinholz, L.H. and Keller, R. (1973) Gen. Comp. Endocr. 21, 554–564.
[14] Huberman, A. and Aguilar, M.B. (1986) Comp. Biochem. Physiol. 85B, 197–203.
[15] Soyez, D. and Van Deijnen, J.E. (1987) J. Exp. Zool. 244, 479–484.
[16] Chang, E.S., Bruce, M.J. and Newcomb, R.W. (1987) Gen. Comp. Endocr. 65, 56–64.
[17] Meusy, J.J., Martin, G., Soyez, D., Van Deijnen, J.E. and Gallo, J.M. (1987) Gen. Comp. Endocr. 67, 333–341.
[18] Quackenbush, L.S. and Herrnkind, W.F. (1983) J. Crust. Biol. 3, 34–44.
[19] Marti, T., Takio, K., Walsh, K.A., Terzi, G. and Truman, J.W. (1987) FEBS Lett. 219, 415–418.