AMINO ACID SEQUENCES AROUND THE CYSTINE RESIDUES IN EQUINE GROWTH HORMONE

M.M. ZAKIN, E. POSKUS, J.M. DELLACHA, A.C. PALADINI and J.A. SANTOMÉ

Facultad de Farmacia y Bioquímica, Departamento de Química Biológica y Centro para el Estudio de las Hormonas Hipofisarias, Junín 956, Buenos Aires, Argentina.

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1. Introduction

Mammalian growth hormones are a family of very similar proteins, as judged by a variety of chemical and physicochemical measurements [1]. This observation is further substantiated by the high degree of homology shown by the primary structures of human [2] and bovine [3] growth hormones, as well as by fragments of known sequence from ovine [4 6] and porcine [7] hormones. The only recorded exception is equine growth hormone which, according to Oliver and Hartree [8], has one disulphide bridge of the expected size and structure as in all these proteins, while the second one joins only 6 amino acid residues instead of the approx. 100 units found in the human and bovine hormones. From an evolutionary standpoint, this variation in structure is of such magnitude as to warrant independent confirmation. In the present paper we report a re-investigation of this problem.

2. Material and methods

2.1. Equine growth hormone was prepared by the procedure of Conde et al. [9]. The protein was homogeneous as judged by several criteria such as ultracentrifugation, disc electrophoresis, gel filtration and N- and C-terminal amino acid estimations. Its biological activity was 1.4 USP units per mg, measured by the tibia test [10]. No prolactin activity was detectable by the pigeon crop sac assay [11]. The highest dose of equine growth hormone injected was 250 μg, while the assay clearly detected 20 μg of ovine prolactin (2nd. International Standard, 22 I.U./mg). TPCK-treated trypsin and pepsin were from Worthington Biochemica Corp., USA, and carboxypeptidase B from Schwarz/Mann, USA. Sephadex was obtained from Pharmacia, Uppsala, Sweden, and Dowex 50W X2 from J.T. Baker Chemical Corp., USA.

2.2. Amino acid analysis and sequence determinations. Peptides were hydrolyzed as described by Wolfenstein et al. [12]; a Technicon TSM-1 autoanalyzer was used for the amino acid analyses. Carboxypeptidase B digestions were performed as described by Santome et al. [13]. Edman degradations [14] were carried out by the technique of Elzinga [15] with a modified coupling buffer (equal volumes of pyridine and water, plus 1% triethylamine and ammonium formate to a final concentration of 1 mM). At the end of the coupling reaction pyridine was added until a ratio pyridine/water of 3 to 1 was achieved. In favourable cases the electrophoretic mobility was used to distinguish between asparagine or aspartic acid, and glutamine or glutamic acid-containing peptides.

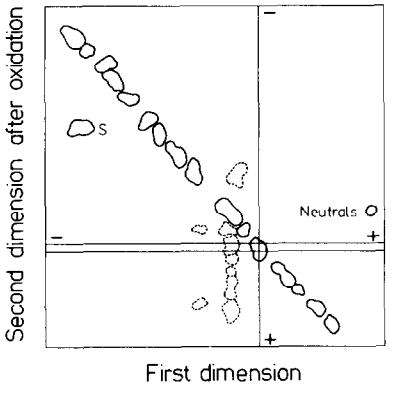

Fig. 1. Diagonal electrophoresis of cystine peptides at pH 6.5 from a peptic digest of equine growth hormone. Dotted lines represent impure peptides. The analysis of peptide S is presented in sect. 3.1 and fig.5.

2.3. Diagonal paper electrophoresis for cystine peptides was performed by the method of Brown and Hartley [16].

2.4. Oxidation of equine growth hormone was carried out as indicated by Wolfenstein et al. [12].

2.5. Aminoethylation of equine growth hormone. Reduction was performed by the method of Crestfield et al. [17] and aminoethylation by the method of Raftery and Cole [18].

2.6. Tryptic peptide maps. The oxidized and aminoethylated hormones were digested with trypsin as indicated by Seavey et al. [19] with a 1:66 weight ratio of enzyme to protein. Peptide mapping was made according to Katz et al. [20], but the electrophoresis was carried out at pH 6.5. Peptides, located with 0.02% ninhydrin solution in acetone, were eluted with 6 M HCl of 1% acetic acid and the solutions used for amino acid analysis or Edman degradations, respectively.

2.7. Ascending two-way paper chromatography. The first run was carried out overnight with a butanol–ammonia–water–ethanol solvent (50:2:18:15; v/v), and the second run at right angles overday, with a butanol–formic acid–water solvent (75:15:10; v/v).

2.8. Peptic digestion of equine growth hormone was performed at 37° for 16 hr in 5% formic acid with a 1:40 weight ratio pepsin to hormone.

2.9. Gel filtration on Sephadex G-50. The peptic digest obtained with 30 mg of hormone was dissolved in 2 ml of 0.6 M ammonium hydroxide and filtered through a Sephadex G-50 column (1.5 cm × 90 cm) equilibrated and eluted with the same solvent at a rate of 10 ml per hr; 3 ml fractions were collected. The cysteic acid concentration in each of the first 30 fractions was established by using 300 μl aliquots which were oxidized as indicated in sect. 2.4, and then submitted to acid hydrolysis.

2.10. Fractionation on Dowex 50W X2. The selected fractions from the Sephadex gel filtration experiment were pooled, oxidized and then dissolved in 0.17 M pyridine-acetic acid buffer, pH 4.8 and submitted to a chromatography on a 0.4 cm × 6 cm column of Dowex 50W X2, 50–100 mesh. Elution was performed with 0.17 M, pH 4.8 and 8.0 M, pH 5.6, pyridine–acetic acid buffers followed by water and concentrated ammonia. One ml fractions were collected and amino acid determinations carried out on 100 μl aliquots.

2.11. Partial acid hydrolysis. Usually 0.2 μmole of peptide was dissolved in 500 μl of 12 M HCl and incubated at 40° for 21 hr.

3. Results

3.1. *Diagonal paper electrophoresis for cystine peptides*

The diagonal map of the cystine peptides from a peptic digest of equine growth hormone shown in fig.1 is similar to the corresponding bovine growth hormone map [21]. The amino acid composition of peptide S, expressed as uncorrected molar ratios obtained after 20 hr of hydrolysis, is:
$CysSO_3H_{2.2}MetSO_{2\,1.0}Ser_{2.0}Glu_{1.1}Ala_{1.0}Val_{1.6}$
$Leu_{0.4}Phe_{2.0}Lys_{1.2}Arg_{3.0}$.

3.2. *Tryptic peptide map of oxidized equine growth hormone*

This map is shown in fig.2. The amino acid com-

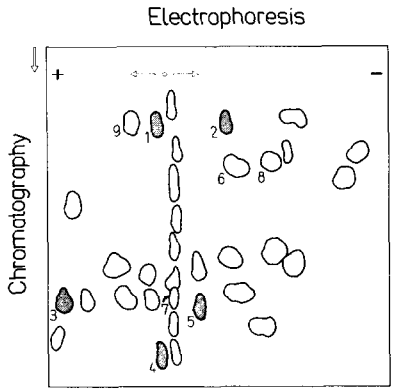

Fig. 2. Tryptic map of oxidized equine growth hormone. Cysteic acid peptides are shaded (see table 1).

position of cysteic acid peptides and some other tryptic fragments is given in table 1.

3.3. *Tryptic peptide map of aminoethylated equine growth hormone*

This map is shown in fig.3. Its comparison with the map in fig.2 indicates the appearance of new spots. Peptide B was further purified by ascending two-way paper chromatography. The amino acid composition and Edman degradation results from A,B,C and D are given in table 2. Digestion of peptide B with carboxypeptidase B for 15 min released aminoethylcysteine and phenylalanine.

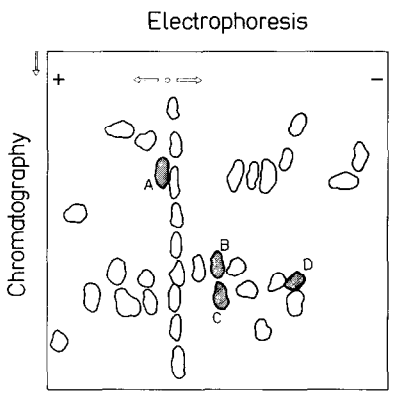

Fig. 3. Tryptic map of aminoethylated equine growth hormone. Peptides A,B,C and D (see table 2) are not present in the tryptic map of the oxidized hormone (fig.2).

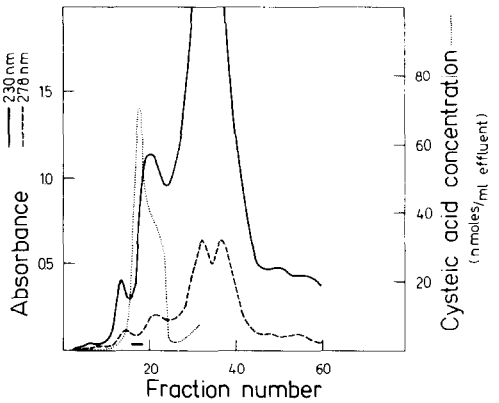

Fig. 4. Gel filtration on Sephadex G-50 of a peptic digest of equine growth hormone. The fractions pooled are indicated by (—) (see table 3).

Table 1
Amino acid composition of selected tryptic fragments from oxidized horse growth hormone.

Peptide no.	Amino acid composition
1	$CySO_3H_{1.0}Arg_{1.0}$
2	$CySO_3H_{1.0}Arg_{1.6}$
3	$CySO_3H_{1.1}Ser_{1.7}Glu_{1.0}Ala_{0.9}Val_{0.8}Phe_{1.7}$
4	$CySO_3H_{0.9}Asp_{0.8}Ser_{1.1}Gly_{1.1}Leu_{2.1}$ $Tyr_{0.9}Phe_{1.1}Lys_{0.8}$
5	$CySO_3H_{1.1}Asp_{0.8}Ser_{1.0}Gly_{1.1}Leu_{2.2}$ $Tyr_{1.0}Phe_{1.1}Lys_{1.5}$
6	$Asp_{0.8}Leu_{1.1}His_{0.8}Lys_{1.0}$
7	$Thr_{1.1}Glu_{1.1}Ala_{0.6}Leu_{1.0}Tyr_{1.0}Arg_{0.7}$
8	$Val_{1.0}MetSO_{20.7}Lys_{1.0}$
9	$Asp_{0.7}Glu_{3.1}Ala_{1.1}Arg_{1.1}$

The numbers correspondent to those in fig.2. Peptides 6,7,8 and 9 are related to the structure around the disulphide bridges.

3.4. *Cysteic acid peptides from a peptic digest of equine growth hormone*

The elution pattern of a peptic digest of the native hormone filtered through a Sephadex G-50 column is shown in fig.4. The material contained in fractions 17 and 18 was oxidized and afterwards chroma-

Table 2
Amino acid composition and Edman degradation of selected tryptic fragments from aminoethylated horse growth hormone.

Peptide	Step	Substractive method Amino acid (moles/mole of peptide)										
		Asp	Thr	Ser	Glu	Pro	Gly	Ala	Ile	Phe	Lys	Arg
A	0	0.9	2.1	1.0	4.0	1.6	1.2	1.5	0.8	0.7	0.8	0.7
	1	1.0	2.0	1.0	4.0	1.8	1.2	1.7	0.9	0.3	0.9	N.D.
	2	1.1	2.2	0.6	4.1	1.8	1.1	1.6	0.8	Tr	N.D.	N.D.
	3	1.3	2.1	Tr	3.2	1.6	1.0	1.6	0.9	Tr	N.D.	N.D.
	4	1.3	1.6	Tr	2.9	1.8	1.0	1.4	0.8	Tr	N.D.	N.D.
	5	1.0	1.6	Tr	2.8	1.6	0.9	1.4	0.3	Tr	N.D.	N.D.
	6	1.3	1.7	Tr	2.8	1.3	0.9	1.3	0.3	Tr	N.D.	N.D.
		Asp	Ser	Glu	Ala	Ile	Tyr	Phe	AECys			
B	0	1.0	1.1	2.1	2.7	0.8	0.7	1.1	+			
	1	1.2	1.1	2.3	2.5	0.7	0.4	0.9	+			
	2	1.0	0.7	2.3	2.5	0.9	Tr	1.1	+			
		Asp	Ser	Gly	Leu	Tyr	AECys					
C	0	0.7	1.1	1.1	2.2	0.9	+					
	1	0.1	0.9	1.1	2.0	1.0	+					
	2	0.2	0.9	1.2	1.9	0.5	+					
	3	0.2	1.1	0.7	1.9	0.2	+					
		Phe	Lys									
D	0	1.0	1.0									

The letters correspondent to those in fig.3. N.D.: not determined; Tr: traces. The underlined values refer to the amino acid assumed to be lost at the indicated degradation step.

Table 3
Amino acid composition and Edman degradation of the cysteic and peptides obtained from a peptic digest of horse growth hormone (fig.4).

Peptide	Step	Subtractive method Amino acid (moles/mole of peptide)									
		$CySO_3H$	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Ile	Phe	Lys
Acidic	0	0.9	1.1	1.9	1.2	3.1	1.7	1.4	1.8	0.8	1.3	1.1
	1	0.3	1.1	1.9	1.2	3.1	2.1	1.4	1.7	0.8	1.4	1.1
		$CySO_3H$	Asp	Thr	Ser	Glu	Ala	Leu	Tyr	Phe	His	Lys
Basic	0	0.6	1.0	0.9	0.9	1.3	1.0	2.0	0.8	1.0	0.9	2.6
	1	0.7	0.9	1.1	0.8	1.2	1.0	1.0	0.7	1.0	N.D.	N.D.
	2	0.7	1.0	1.1	0.5	1.3	1.0	1.3	0.7	1.0	N.D.	N.D.
	3	0.4	1.0	1.1	0.5	1.2	1.0	1.0	1.0	0.8	N.D.	N.D.

N.D.: not determined. The underlined values refer to the amino acid assumed to be lost at the indicated degradation step.

tographed on a column of Dowex 50W X2. Only two major peaks were obtained; one of them, containing an acidic peptide, was eluted with pyridine–acetic acid buffer 0.17 M pH 4.8 and the other one, with a very basic peptide, was eluted with concentrated ammonia. Amino acid composition and Edman degradation results from these peptides are given in table 3. The acidic fragment was submitted to partial acid

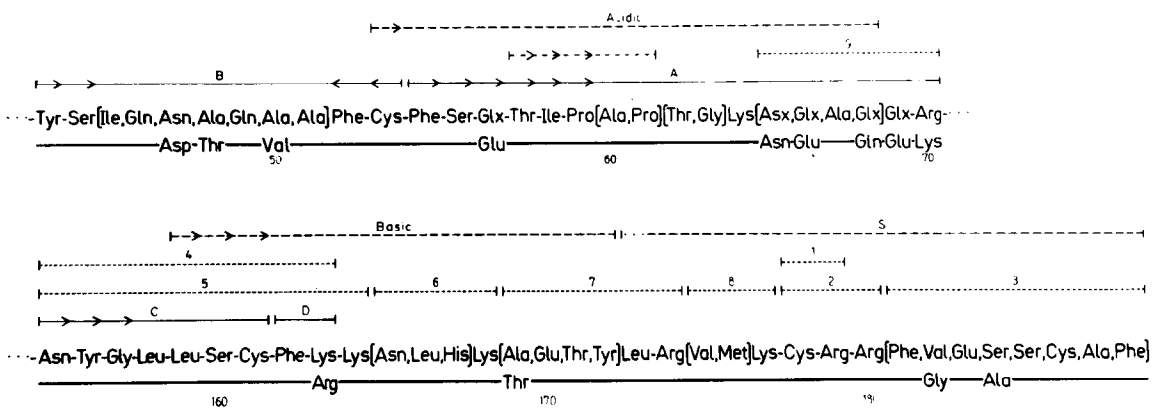

Fig. 5. Proposed structure of equine growth hormone around the disulphide bridges. The sequences of the groups of amino acids in parentheses are unknown except that of peptide 3, which was established by Oliver and Hartree [8]; in all other cases the sequences shown are tentative.- - - -, tryptic peptides of oxidized protein;——, tryptic peptides of aminoethylated protein; – – –, peptic peptides;–·–·–, peptides from partial acid hydrolysis;——, identical sequence in bovine growth hormone; differences are indicated. →, Steps of Edman degradation; ←, residues released by carboxypeptidase B.

Table 4

Amino acid composition and Edman degradation of a pentapeptide isolated after partial acid hydrolysis of the acidic cysteic acid peptide in table 3.

Step	Subtractive method Amino acid (moles/mole of peptide)			
	Thr	Pro	Ala	Ile
0	0.7	2.0	1.0	1.0
1	0.3	2.0	1.2	1.0
2	0.3	2.0	1.0	0.7
3	Tr	1.6	1.0	0.6

Tr: traces. The underlined values refer to the amino acid assumed to be lost at the indicated degradation step.

hydrolysis and the product was fractionated by ascending two-way paper chromatography. A pentapeptide was isolated and its amino acid composition as well as the results of its Edman degradation are indicated in table 4.

4. Discussion

All the information collected about the disulphide bridges in equine growth hormone is organized in fig.5 to give unique sequences of amino acids. The composition of peptide S agrees well with the C-terminal amino acid sequence reported by Oliver and Hartree [8] except for the leucine value. The low molar proportion obtained for this amino acid can be explained if we accept its N-terminal position in S, as suggested by homology with the structure of this region of the molecule in bovine growth hormone. and a peptic splitting of the hormone on both sides of the leucine residue. S would actually be a mixture of two almost identical peptides unresolved in the diagonal map. The amino acid compositions of peptides 1,2,3 and 8 are consistent with that of peptide S (table 1). Peptide 7 overlaps both S and the basic peptide described in table 3. Cysteic acid peptides 4 and 5 found in the tryptic peptide map of the oxidized hormone (fig.2; table 1) must obviously belong to the second disulphide bridge in the molecule. Peptides C and D (fig.3; table 2) are fragments of peptides 4 and 5. The basic peptide overlaps peptides C and D and tryptic fragments 4,5,6 and 7. This evidence gives support to a unique sequence 34 amino acids long, which includes a disulphide bridge and contains a half cystine residue. Although the complete primary structure of this fragment has not been established there is no doubt that it is highly homologous with same region of the molecule in bovine growth hormone (fig.5) and in porcine growth hormone (7). Peptide B (fig.3, table 2) is a tryptic fragment containing aminoethylcysteine which, according to the specificity of the

enzyme, must be located in its C-terminal end. The acidic peptide (table 3) contains a half cystine residue, as cysteic acid, in its N-terminus, and overlaps tryptic fragments A and B. This evidence gives support to a sequence of 28 amino acids that is highly homologous to the region in bovine growth hormone comprised between amino acid residues 43 and 70. The structure of peptide 9 (table 1) and of the pentapeptide isolated after partial hydrolysis of the acidic peptide (table 4), fit in with the proposed sequence. The second disulphide bridge in horse growth hormone must arise by joining the half cystine residues in the two polypeptide chains described (fig. 5). This conclusion is supported by the fact of the acidic and basic cysteic acid peptides being obtained simultaneously after oxidation of a purified fraction from a peptic digest of the native hormone. It is evident that there is in equine growth hormone a high degree of homology with bovine growth hormone throughout the whole region containing the disulphide bridges. We have found no indication of the disulphide bridge described by Oliver and Hartree [8] in their preparation of equine growth hormone. It is very suggestive though, that the primary structure of the Oliver and Hartree peptide strongly resembles that of the C-terminal disulphide bridge in ovine prolactin [22]. Since prolactin is a possible contaminant in growth hormone preparations, it seems likely that the hormone used by these authors was contaminated with equine prolactin.

Acknowledgements

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