REACTIVITY AND BIOLOGICAL IMPORTANCE OF THE DISULFIDE BONDS IN HUMAN GROWTH HORMONE

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1. Introduction

Different methods have been reported for complete reduction and alkylation of the two disulfide bonds in the structure of human growth hormone (HGH) [1–4]. More recent studies on partial reduction and alkylation of the disulfide bonds in porcine [5], human [6] and bovine growth hormones [7] indicated that the small disulfide loop near the COOH-terminus in these hormones is much more exposed to reducing agents than the large loop. It has been reported that the completely reduced and carbamidomethylated derivative of HGH retains the full biological potency of the native hormone [1–4], whereas complete reduction and carboxymethylation of the disulfide bonds in HGH leads to a considerable loss of the biological activities [4]. In view of this latter observation it has been of interest to attempt the selective reduction and carboxymethylation of the COOH-terminal disulfide loop of HGH, and to determine the effect of this modification on the biological properties of HGH. In this paper we report the preparation and characterization of reduced di-*S*(182,189)-carboxymethylated HGH.

2. Experimental

2.1. *Materials*

HGH was prepared by the method of Gráf et al. [8]. [2-^{14}C]bromoacetic acid (5.73 mCi/mmol) was prepared in the isotope laboratory of our institute. Dithiothreitol, iodoacetic acid, iodoacetamide and trypsin were obtained from Calbiochem.

2.2. *Reduction and carboxymethylation of HGH; reactivity of disulfide bonds*

HGH was reduced with different molar excesses of dithiothreitol over protein cystine content in 0.1 M Tris buffer, pH 8.3, in the presence or absence of 8 M urea under nitrogen at room temperature for 40 min with a protein concentration of 5 mg/ml. Alkylation was carried out under the same conditions for 10 min by adding [2-^{14}C]bromoacetate in 10-fold molar amount to dithiothreitol. The reaction mixture was extensively dialysed against distilled water, and then freeze dried.

The degree of reduction and carboxymethylation of the disulfide bonds was assessed by acid hydrolysis (6 M HCl, 24 h, 110°C, sealed, evacuated tubes) and amino acid analysis carried out in a JEOL (JLC-5AH) amino acid analyzer.

The reduced and carboxymethylated derivatives of HGH were digested with trypsin in 0.05 M ammonium acetate buffer, pH 7.5, with an enzyme to protein ratio of 1:50 (w/w) at 37°C for 2 h. Two mg samples of the hydrolysates were mapped by two-dimensional high voltage paper electrophoresis, first at pH 5.0 (pyridine–acetic acid–water, 10:10:1000 by vol), and then at pH 2.0 (formic acid–acetic acid–water, 50:150:800 by vol). Radioautographs were obtained from the peptide maps on Forte X-ray film with an exposition of 60 h. Relative radioactivity of the fragments was estimated by densitometry of the radioautographs in a Zeiss (Jena) densitometer. The radioactive peptides were cut out from the maps and eluted with 0.5 M ammonium hydroxide solution. The dried eluates were hydrolysed for amino acid analysis.

2.3. *Preparation and characterization of reduced tetra-S- and di-S-alkylated derivatives of HGH*

Reduced and tetra-*S*-carboxymethylated and tetra-*S*-carbamidomethylated derivatives of HGH were prepared by two alternative procedures: (A) in the presence of 8 M urea as described by Dixon and Li [1], (B) and in the absence of urea according to Bewley et al. [2]. Partially reduced and carboxymethylated HGH was prepared in the absence of urea with 4 M excess of dithiothreitol over the cystine content in HGH according to the procedure described in section 2.2. The product obtained was subjected to chromatography on a DEAE-cellulose column.

Gel electrophoresis was performed at pH 9.0 in 8% polyacrylamide gel by the method of Davis [9]. The growth promoting and prolactin activities were determined by the rat tibia test [10] and the quantitative pigeon crop-sac assay [11], respectively.

3. Results

3.1. *Reactivity of the disulfide bonds in HGH*

Table 1 presents the course of reduction and alkylation with increasing amounts of dithiothreitol in the presence and in the absence of 8 M urea. It may be seen from table 1 that the accessibility of the disulfide bonds for reduction and alkylation are considerably restricted in the native conformation. To establish whether or not this steric hindrance of reduction affects the two disulfide bonds uniformly, a series of reduced-carboxymethylated HGH derivatives was digested with trypsin and mapped by two-dimensional paper electrophoresis. As an example, the radioautographs obtained from two different reduced-carboxymethylated HGH derivatives are shown in fig.1. Both these derivatives were reduced-carboxymethylated to 25% (containing one mole of carboxymethylcysteine per mole of HGH), one of them in the presence (fig.1A), the other in the absence of urea (fig.1B). In 8 M urea all the four cysteinyl peptides were uniformly labeled indicating an identical or closely similar reactivity of the two disulfide bonds towards dithiothreitol (fig.1A). In the absence of urea most of the radioactivity was found in fragments C_1 and C_2 (fig.1B).

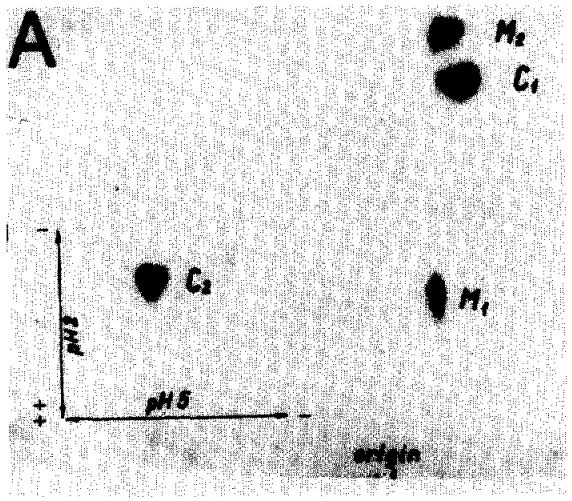
The amino acid analysis of the radioactive peptides gave evidence that fragments C_1 and C_2 are derived from residues 179–183 and 184–191 of the sequence, respectively, whereas fragments M_1 and M_2 came from sequence regions of residues 42–64 and 165–167, respectively (for sequence see [12]).

The above data indicate that the disulfide bond between Cys_{182}–Cys_{189} is much more exposed to reduction than the disulfide bridge between Cys_{53}–Cys_{165} of the sequence. Semiquantitative evaluation of the radioautographs obtained from tryptic peptide maps of partially reduced and carboxymethylated HGH derivatives prepared in the absence of urea with increasing amounts of dithiothreitol, showed that the reactivity of the COOH-terminal disulfide bond is five to six times higher than that of the other disulfide bond.

Table 1
Reactivity of the disulfide bonds in HGH with increasing amounts of dithiothreitol

Dithiothreitol disulfide bond[a]	Carboxymethylcysteine content[b] of reduced and carboxymethylated HGH derivatives prepared in:	
	the presence of urea	the absence of urea
1	1.0	0.2
2	2.5	0.9
3	3.1	1.5
4	3.7	2.3
6	3.9	2.8

[a] Molar ratio
[b] Moles of carboxymethylcysteine per mole of HGH as determined by amino acid analysis



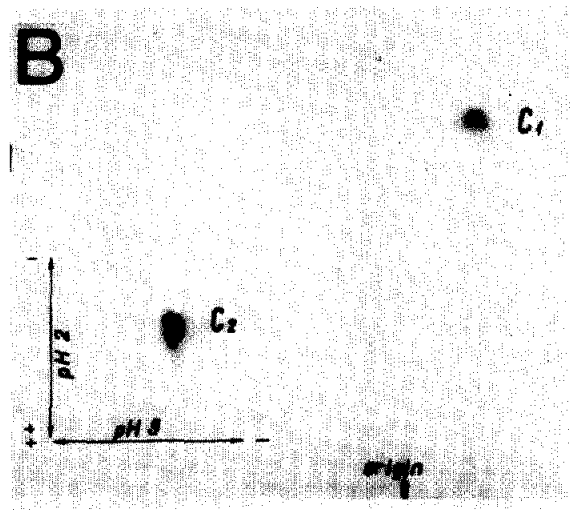


Fig.1. Radioautographs of the tryptic peptide maps of two partially (to 25%) reduced and carboxymethylated HGH derivatives prepared (A) in the presence and (B) in the absence of 8 M urea.

3.2. *Preparation and characterization of reduced and di-S(182,189)-carboxymethylated HGH*

HGH was partially reduced with 4 M excess of dithiothreitol over the HGH cystine content in the absence of urea, and carboxymethylated as described in section 2.2. This product was submitted to DEAE-cellulose chromatography as shown in fig.2. Fig.3 shows the gel electrophoretic patterns of the partially reduced and carboxymethylated HGH (tube a), its fractions obtained from the DEAE-cellulose column (tubes b–d), intact HGH (tube e), and two completely reduced and carboxymethylated HGHs (tubes f and g). It may be seen in the figure that fraction 1 of fig.2 is a mixture, fraction 3 is mainly composed of tetra-*S*-carboxymethylated HGH, but fraction 2 is essentially free of native and tetra-*S*-carboxymethylated HGHs. Hence fraction 2 was further characterized. Its amino acid analysis revealed the presence of 2.2 carboxymethylcysteine per mole of HGH. Fraction 2 was shown to consist of 94% of di-*S*(182,189)-carboxymethylated HGH and 6% of di-*S*(53,165)-carboxymethylated HGH, as inferred from the radioautograph of its tryptic peptide map.

The biological potencies of the native and different reduced-alkylated HGH preparations are summarized in table 2.

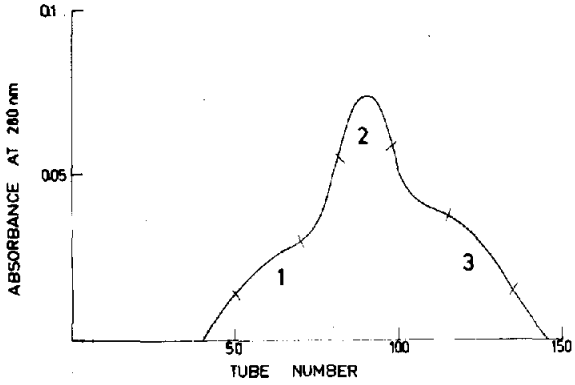


Fig.2. Chromatography of 50 mg of partially reduced and carboxymethylated HGH (see section 3.2.) on DEAE-cellulose (15 × 1.5 cm). The column was equilibrated with 0.05 ammonium hydrogencarbonate, pH 8.0, and the elution was performed by adding 0.3 M ammonium hydrogencarbonate, pH 8.0, to a 500 ml mixing flask which contained the starting buffer; tube volume, 5 ml; 40 ml/h. The indicated fractions were pooled and lyophilized. The yields of fractions 1, 2 and 3 were 6.4 mg, 13.2 mg and 8.7 mg, resp..

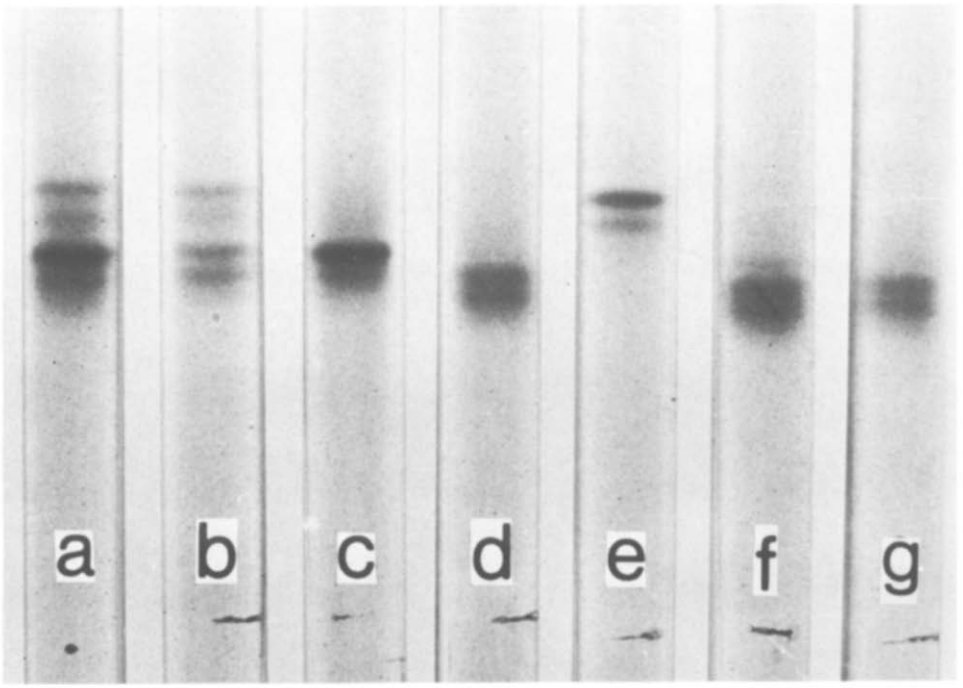


Fig.3. Disc electrophoresis of partially reduced and carboxymethylated HGH (a), the fractions of this preparation (fig.2), fraction 1 (b), fraction 2 (c), fraction 3 (d), and native HGH (e) and completely reduced and carboxymethylated HGHs prepared in the absence (f; see [2]) and in the presence of urea (g; see [1]); 60 μg samples.

Table 2
Biological potency of reduced and alkylated derivatives of HGH as measured by rat tibia and pigeon crop-sac assays

Material[a]	Rat tibia test		Pigeon crop-sac test	
	Total dose (μg)	Tibia width[b] (μm)	Total dose (μg)	Dry mucosal weight[b] (mg)
Saline	0	154 ± 2 (6)	0	9.0 ± 2.0 (6)
HGH	25	195 ± 6 (5)	4	16.1 ± 2.1 (6)
	50	253 ± 3 (5)	10	20.1 ± 1.9 (6)
			25	22.7 ± 1.9 (5)
di-*S*(182,189)-carboxymethylated HGH	25	205 ± 8 (6)	4	17.8 ± 3.0 (4)
	50	235 ± 6 (6)	25	21.0 ± 1.4 (4)
tetra-*S*-carboxymethylated HGH [1]	50	147 ± 5 (5)	Not measured	
	150	160 ± 9 (4)		
tetra-*S*-carbamidomethylated HGH [1]	25	190 ± 5 (5)	4	15.6 ± 2.4 (4)
	50	245 ± 2 (6)	25	20.2 ± 2.2 (4)
tetra-*S*-carboxymethylated HGH [2]	25	160 ± 3 (5)	4	11.6 ± 1.2 (5)
	50	185 ± 4 (6)	10	13.0 ± 2.0 (4)
	100	212 ± 3 (3)	25	15.6 ± 1.3 (5)

[a] di-*S*(182,189)-carboxymethylated HGH was obtained from fraction 2 of fig.2.; tetra-*S*-alkylated HGHs were prepared according to Dixon and Li [1] and Bewley et al. [2].

[b] Mean ± s.e.m.; number of animals is indicated in parentheses.

3. Discussion

It is apparent from table 2 that reduced and di-*S*(182,189)-carboxymethylated HGH retains the full biological potency of native HGH as measured by the rat tibia and pigeon crop-sac tests. Our assay data on the completely reduced and alkylated HGH derivatives confirm the earlier results of Li and co-workers [1–4]: complete reduction and carboxymethylation of both disulfide bonds in HGH abolishes the growth promoting activity of the hormone [3], whereas reduction and carbamidomethylation of the disulfide bridges do not alter the biological properties of HGH [1–4]. The slight biological activity of tetra-*S*-carboxymethylated HGH prepared according to Bewley et al. [2] (table 2) may be due to the presence of a small amount of contaminating di-*S*(182,189)-carboxymethylated HGH in this preparation (see tube f in fig.3).

The full biological potency of di-*S*(182,189)-carboxymethylated HGH together with the abolished activity of tetra-*S*-carboxymethylated HGH suggest that the reduction and carboxymethylation of the disulfide bond between Cys_{53}–Cys_{165} in the HGH structure is responsible for the loss of biological activity. The inactivating effect of the carboxymethyl groups introduced on the cysteinyl residues at sequence positions 53 and 165 of HGH may be due to an electrostatic repulsion between these groups which weakens or destroys the interaction between the two cysteine-containing portions of the molecule. The importance of such interaction(s) for the full biological potency of HGH has also beer pointed out by Li and Gráf [13].

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