CARBOXYMETHYLATION OF METHIONINE RESIDUES IN BOVINE GROWTH HORMONE

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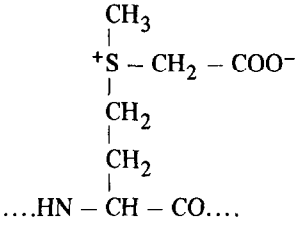
1. Introduction

The primary structure of bovine growth hormone (BGH) has been studied by several groups over the last few years [1–5]. The hormone possesses a single polypeptide chain containing 4 methionine residues, the approximate positions of which are indicated in fig. 1. This paper describes studies on the reaction of these residues with iodoacetic acid. The investigation was designed to determine the importance of methionine for the biological activity of the hormone and to assess the relative reactivities of the 4 residues of this amino acid. Carboxymethylation was carried out at pH 3.5, at which pH only methionine residues should react [6]; they are converted to S-carboxymethylmethionine:



The results show that carboxymethylation of all the methionine residues in BGH leads to almost complete loss of growth promoting activity. The 4 residues differ considerably in their reactivities, residue II (fig. 1) being least readily carboxymethylated.

2. Experimental

2.1. *Materials*

BGH was prepared by the method of Wallis and Dixon [7]. 2-^{14}C-iodoacetic acid (specific activity 7 mCi/mmole) was obtained from the Radiochemical Centre (Amersham, Bucks). Trypsin (TPCK-treated) and α-chymotrypsin (3 × crystallized) were obtained from Worthington Biochemical Corporation.

2.2. *Preparation and characterization of carboxymethyl-bovine growth hormone*

BGH was dissolved in formate/formic acid buffer, containing urea (0.2 M sodium formate, 8 M urea, pH 3.5) to give 8 mg protein/ml, and recrystallized iodoacetic acid (100 μmole of protein; a 25-fold molar excess over methionine residues) was added and dissolved. The mixture was incubated at 37° for 24 hr, and then dialysed against distilled water and freeze dried. As a control, a second sample of BGH was treated in the same way, but without the addition of iodoacetic acid.

The degree of carboxymethylation of methionine cannot be easily assessed by acid hydrolysis and amino acid analysis, because S-carboxymethylmethionine is readily degraded [6]. The protein was therefore subjected to performic acid oxidation [8] and then hydrolysed (0.68 mg protein, 0.2 ml 6 N HCl, 110°, 24 hr) and analysed on a Locarte amino acid analyser. Complete absence of methionine sulphone from the hydrolysate indicated that all the methionine of the original BGH had been carboxymethylated [6, 9].

The growth promoting activity of carboxymethylated BGH was assayed in pituitary dwarf mice (Snell's strain) [10, 11], and compared with that of the control BGH

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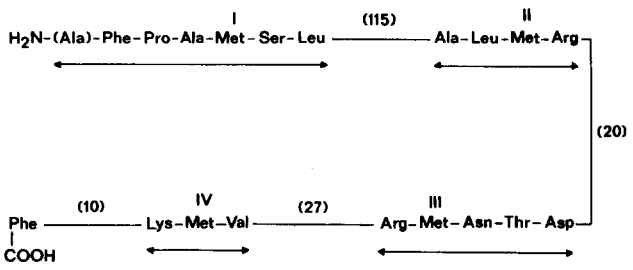

Fig. 1. Amino acid sequences around the 4 methionine residues of bovine growth hormone. The numbers in parentheses give the approximate number of residues in the regions between the sequences shown. Double-headed arrows below the sequence show the peptides isolated in the present work. The N-terminal Ala residue is in parentheses because it is missing in about half the molecules [4]. The figure is based on the data of [1–5] and on the present studies.

sample which had been treated with urea but not iodoacetic acid.

2.3. *Preparation of ^{14}C-labelled carboxymethyl-bovine growth hormone; reactivity of methionine residues*

BGH was dissolved in the same formate/formic acid buffer as before, at a concentration of 8 mg/ml. In studies involving the effect of a denaturing agent on the reactivity of the methionine residues, 8 M urea was included in the reaction mixture, otherwise it was omitted. Sufficient 2-^{14}C-iodoacetic acid was added to give a 2-fold molar excess of iodoacetic acid over protein (0.5 μmole of iodoacetic acid/μmole of methionine). The mixture was incubated for 24 hr at 37°, and then excess unlabelled (recrystallized) iodoacetic acid was added to give 100 μmoles/μmole of protein (a 25-fold molar excess over methionine). Urea, if not already present, was added to give a final concentration of about 8 M. The mixture was incubated for a further 24 hr at 37°, and then dialysed against distilled water and freeze dried. The conditions used for the second incubation give complete carboxymethylation of the methionine residues in BGH (see sect. 2.2).

^{14}C-carboxymethyl-BGH was digested with trypsin and chymotrypsin. 4 mg of the modified hormone were suspended in 1 ml *N*-ethylmorpholine/acetic acid buffer (0.2 M in *N*-ethylmorpholine, pH 8.5) and digested with 80 μg of TPCK-treated trypsin (3 hr, 37°), and then 80 μg of chymotrypsin (3 hr, 37°). The digest was freeze dried.

Peptides were separated on a peptide map (2 mg of digest/map) by 2-dimensional high voltage paper electrophoresis [12] (first dimension: pH 6.5, 10% pyridine, 0.3% acetic acid. 89.7% water; 55 min, 2.5 kV; second dimension: pH 2, 8% acetic acid, 2% formic acid, 90% water, 70 min, 2.5 kV). The position of radioactive peptides was detected by autoradiography (Kodak 'Kodirex' X-ray film; 4 days). The radioactive peptide spots that were positively or negatively charged at pH 6.5 were cut out and eluted with pH 2 buffer. The region of the peptide map containing peptides neutral at pH 6.5 (within which separation was incomplete) was cut out, sewn onto another piece of chromatography paper and chromatographed in n-butanol/acetic acid/water/pyridine (75/15/60/50, by vol). This gave a map of the neutral peptides. Again, radioactive peptides were detected by autoradiography, and eluted with pH 2 electrophoresis buffer.

Samples of the radioactive peptides ($\frac{1}{3}$) were taken for hydrolysis (6 N HCl, 110°, 24 hr, sealed evacuated tubes, plus a trace of phenol) and amino acid analysis on a Locarte amino acid analyser. Samples were also taken for scintillation counting ($\frac{1}{100}$) – these were dried onto glass fibre discs and counted in a Beckman scintillation counter in toluene containing 0.35% 2,5-diphenyloxazole, and 0.005% 1,4-bis-(5-phenyloxazol-2-yl) benzene dimethyl ester.

From the information available about the amino acid sequence of BGH [1–5] and the amino acid compositions of the peptides isolated, it was possible to assign each radioactive peptide to a sequence around a methionine residue. Further sequence studies have confirmed these assignments. No evidence was obtained for modification of any residue other than methionine. The specific activity (dpm/nmole) of each peptide and hence each methionine residue could then be determined. These specific activities reflect the relative reactivities of the different methionine residues, since labelling took place when these residues were competing for a rather small amount of iodoacetic acid during the first stage of the carboxymethylation procedure.

Table 1
Growth promoting activity of carboxymethyl-bovine growth hormone in dwarf mice.

Sample		Number of animals	Weight gain (g)	(± S.E.M.)
Assay I	1. saline controls	5	+ 0.41	(± 0.36)
	2. 2 μg BGH/day	6	+ 1.51*	(± 0.31)
	3. 10 μg BGH/day	5	+ 2.90**	(± 0.40)
	4. 10 μg urea treated BGH/day	4	+ 2.62**	(± 0.20)
	5. 20 μg carboxymethyl-BGH/day	6	+ 0.14	(± 0.25)
Assay II	6. saline controls	6	+ 1.01	(± 0.17)
	7. 2 μg BGH/day	6	+ 1.85**	(± 0.15)
	8. 5 μg BGH/day	6	+ 2.73***	(± 0.28)
	9. 40 μg carboxymethyl-BGH/day	6	+ 1.14	(± 0.21)
	10. 5 μg BGH + 40 μg carboxymethyl-BGH/day	6	+ 3.40***	(± 0.36)

The weight gain shown was the mean increase in weight for each group of mice after 21 (Assay I) or 22 (Assay II) daily injections of the test solution indicated. The volume injected was 0.1 ml/day in every case, and all the samples were dissolved in 0.9% NaCl and adjusted to pH 7 – 9. For the difference between samples and the appropriate controls: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. The differences between 1 and 5, 3 and 4, 6 and 9, and 8 and 10 are not significant.

3. Results

3.1. *Characterization and biological activity of carboxymethyl-bovine growth hormone*

Amino acid analysis of carboxymethyl-BGH prepared as described in sect. 2.2 showed that the composition of the carboxymethylated, performic oxidized hormone was identical to that of performic oxidized BGH except for the complete absence of methionine sulphone. The latter was replaced by the breakdown products of S-carboxymethylmethionine (S-carboxymethylhomocysteine, methionine, homoserine and homoserine lactone [6]). The result indicates that all the methionine groups of the protein had been carboxymethylated, and that no other residues had been modified (though modification of other residues cannot be excluded completely since acid hydrolysis might remove the modifying group).

The growth promoting activity of carboxymethyl-BGH in dwarf mice is shown in table 1. BGH treated with urea (but not iodoacetic acid) under the conditions used for carboxymethylation had growth promoting activity which was not significantly different from that of the untreated hormone (Assay I). Carboxymethyl-BGH however showed no growth promoting activity at a dose of 20 or 40 μg per day, though 2 μg per day of untreated hormone was active. Clearly the activity of the carboxymethylated hormone is less than 5% of that of untreated hormone. Since the latter had a potency of about 2.0 I.U. per mg, the potency of carboxymethyl-BGH is less than 0.1 I.U. per mg.

It was thought possible that carboxymethyl-BGH might inhibit the action of BGH. However, when 40 μg of this derivative was administered with 5 μg of BGH (Assay II) the growth promoting activity was slightly greater than that of 5 μg of BGH given alone (though the difference was not significant). Clearly, there is no evidence here for inhibition.

3.2. *^{14}C-labelled carboxymethyl-BGH and the reactivity of methionine residues*

Four major peptides located by autoradiography were eluted from peptide maps of material labelled in both the presence and the absence of urea. The location of these peptides in the amino acid sequence (determined from the amino acid composition and confirmed in some cases by sequence studies) is indicated in fig. 1. None of these peptides contained more than one

Table 2
Reactivity of the methionine residues of bovine growth hormone.

Residue (see fig. 1)	Specific activity of carboxymethyl-Met residue*	
	No denaturant	8 M urea
Met I	2,910	3,370
Met II	1,030	1,400
Met III	5,920	5,250
Met IV	4,550	5,090

* Specific activities are expressed in dpm/nmole. BGH was reacted with ^{14}C-labelled iodoacetic acid in the presence or absence of 8 M urea.

methionine residue, so the specific activity of the carboxymethylmethionine could be readily determined from the amino acid composition data and the counts. These specific activities are shown in table 2. The specific activities of the different methionine residues gives a measure of their reactivity with iodoacetic acid. Clearly, in the absence of denaturant, there is a considerable difference between the reactivities of the 4 methionine residues, residue II being markedly less reactive than any of the others. In the presence of 8 M urea, the differences are somewhat reduced, though they still remain.

4. Discussion

The amino acid sequences around methionine residues I, III and IV (fig. 1) agree with those reported by other workers [1–3]. The sequence around methionine II differs somewhat from that of Santomé et al. [3], though there is agreement that there is a methionine residue in this region. Further studies on the amino acid sequence of BGH will be reported in detail elsewhere.

The growth promoting activity of BGH in which all the methionine residues have been carboxymethylated is very low (table 1). This may be because at least one of the 4 methionine residues is required for biological activity, but such a conclusion must clearly be tentative at this stage, since carboxymethylation of the hormone is likely to be accompanied by conformational changes which could themselves lead to inactivation. In this respect it is interesting that a peptide of 41 residues corresponding to part of the sequence of human growth hormone not containing methionine has been synthesized chemically, and shows some growth hormone-like activity [13].

The data shown in table 2 does not give absolute values for the reactivities of the methionine groups in BGH, but does give some indication of their relative reactivities. Clearly, methionine residue II is less reactive towards iodoacetic acid than are the others, and the differences in reactivity are only slightly reduced when the carboxymethylation is performed in 8 M urea. Either the reactivity of the methionine residues is not dependant solely on differences due to the conformation of the protein, or (as seems more likely) 8 M urea does not cause complete unfolding of the BGH molecule. Attempts to repeat the experiment using guanidinium chloride as the denaturing agent have been unsuccessful, because the degree of labelling in the presence of this reagent is much lower than in the absence of a denaturant, or in the presence of 8 M urea. It seems possible anyway that the low activity of methionine II in BGH is due to its inaccessibility to the reagent, presumably because it is buried within the 3-dimensional structure of the protein. It is interesting that if the amino acid sequences of bovine, porcine [14] and human [15, 16] growth hormones are compared, only methionine II (of the 4 methionines in BGH) is conserved in all 3 species.

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