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Immunogenicity and Bioactivity of Glucagon, Modified at Methionine-27

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Summary

Porcine glucagon was modified at methionine-27 by methylation or oxidation. Antisera against the glucagon derivatives were obtained. One of these antisera showed a high affinity for glucagon, with no cross-reactivity with gut-GLI 1.

Biological activities of these derivatives were assessed on rat hepatocytes. Both derivatives had the same maximal glucose-mobilising activity as native glucagon, but a decreased potency, suggesting a crucial role of methionine in the binding of glucagon to its hepatic receptor.

Key-Words: *Porcine Glucagon – Glucagon Derivative (Methionine-27) – Immunogenicity – Bioactivity*

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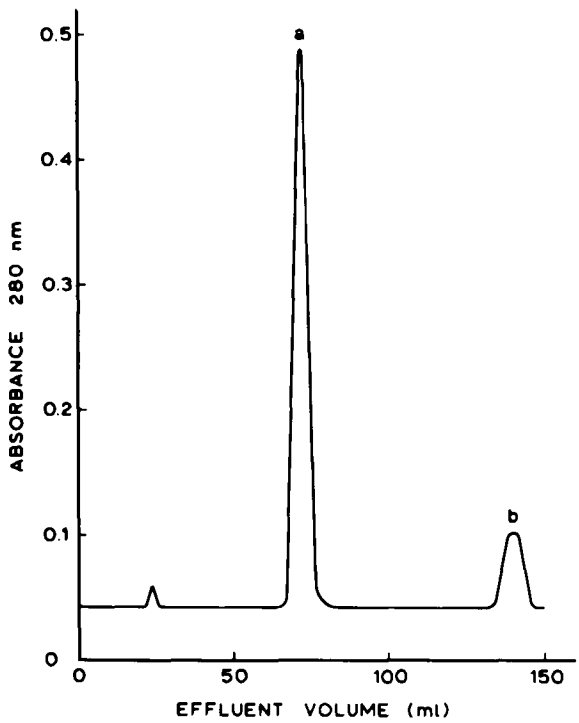

Fig. 1 The purification of s-methylglucagon, demonstrating the separation of s-methylglucagon (a) and glucagon (b)

Introduction

The development of radioimmunoassays for pancreatic glucagon has been hampered by the low immunogenecity of glucagon and by the crossreactivity of most antisera with gut-derived glucagon-like-material (gut GL1), (*Samols* et al. 1966; *Schopman, Hackeng* and *Steendyk* 1967; for review see: *Holst* 1978). As antisera specific for pancreatic glucagon should be directed against the C-terminal part of the molecule (*Assan* and *Slusher* 1972), we reasoned that modification of glucagon at methionine-27might give us a tool for developing a specific glucagon assay. Modification at this residue would make the C-terminal part of the molecule foreign to the immunological recognition system and trigger off the production of antibody, specifically directed against this modified part of the molecule.

In this paper we describe the preparation of the 27-S-methyl and 27-sulfoxide derivatives of glucagon and report the production in rabbits of antisera against these compounds. Having succeeded in preparing such modified glucagon molecules, we thought it of interest to study the bioactivity of these preparations as compared to native glucagon. For this purpose we have used isolated rat hepatocytes, which have been shown to be highly sensitive to glucagon (*Wagle* 1975).

Materials and Methods

Preparation of glucagon derivatives

Porcine glucagon (Lot no. 258-v016-36)was a gift from Messrs. Eli Lilly, Indianapolis, U.S.A. Highly purified gut-GL1 was obtained from Novo Research.

Amino acid analyses were performed by the method of *Spackman* et al. (1958) on a Technicon NCl, equipped with a 65 cm column of C2 resin (Technicon). 27-S-Methylglucagon was prepared in 95–99% yield by reacting 10 mg glucagon with 150 mg methyl-iodide in 10 ml phosphate buffer (0.05 M, 2 M urea; adjusted to 3.5 with 1 N phosphoric acid) at room temperature for 24 hours. The product was purified by ion-exchange chromatography on DEAE-Sephadex A-25 (Fig. 1). On this column the methyl derivative eluted at three times the void volume; traces of unreacted glucagon appeared at six times the void volume.

The degree of methylation was determined as follows. The methylated protein was subjected to performic acid oxidation. By this procedure unmodified methionine residues are converted to methionine sulfone, while the S-methyl-methionine sulfonium iodide remains intact (*Hirs* et al. 1953). Glucagon-27 methionine sulfoxide was prepared by reacting 10 equivalents of chloramine-T with 2 mg/ml glucagon in Tris-hydrochloride buffer (pH 8.5) according to *Shechter, Burnstein* and *Potchornik* (1975). The sulfoxide was separated from degradation products by ion-exchange chromatography on DEAE-cellulose (D.E. 52 Whatman). The degree of oxidation was determined as follows: about 2 mg of the modified glucagon was treated with 0.3 ml 70% formic acid with 5 mg CNBr for 24 hours (*Gross* 1967). Under these conditions any methionine residues are quantitatively converted to homoserine and its lactone while methionine sulfoxide remains intact. The sample is taken to dryness and hydrolysed in 6 N HCl at 110 °C for 22 hours in the presence of about 2 mg dithioerythritol. Under these acid hydrolysis conditions methionine sulfoxide is converted back to methionine and the amount of methione obtained on amino acid analysis represents the amount of methionine sulfoxide originally present. Amino acid analyses of the two derivatives are shown in Table 1.

The tryptophan residue was not affected by the chloramine-T treatment as the molar absorbance at 290 nm was the same before and after treatment.

Production and screening of glucagon antisera

Fifteen female New Zealand white rabbits and three female Wener-Alaska rabbits were immunised with S-methylglucagon. One mg in 50% Freund's adjuvant was given intramuscularly and/or subcutaneously divided over four sites. Boosters were given monthly during eighteen months. Blood was collected on the fourth day after each booster.

The same procedure was applied for glucagon-sulfoxide with two female Werner-Alaska rabbits. The sera were histochemically screened for the presence of antibodies on 4 μ sections of human pancreas (*Nakame* and *Pierce* 1967). Radioimmunoassay was performed using the procedure as described by *Schopman, Hackeng* and *Steendyk* (1967).

Preparation and incubation of rat hepatocytes

Hepatocytes were prepared from the liver of normally fed rats, according to the procedure of *Seglen* (1976), with slight modifications. Immediately after isolation, they were incubated in *Krebs-Henseleit* (1932) medium, buffered with HEPES (instead of bicarbonate) at pH 7.4 under oxygen. Glucagon or its derivatives were dissolved to 10^{-4}M in Krebs-Henseleit medium, at pH 9.0 and serially diluted in Krebs-Henseleit medium with 0.1% bacitracine pH 7.4. One

Table 1 Amino acid compositiion of native glucagon, 27-S-methylglucagon and glucagon-methionine-sulfoxide

Amino acid	No. of amino acid residues per molecule		
	Native glucagon	27-S-methylglucagon	glucagon-methionine-sulfoxide
Aspartic acid	3.9 (4)[a]	3.8 (4)	3.6 (4)
Threonine	3.0 (3)	3.0 (3)	3.2 (3)
Serine	4.1 (4)	3.9 (4)	3.7 (4)
Glutamic acid	2.8 (3)	2.6 (3)	2.9 (3)
Glycine	1.2 (1)	1.1 (1)	1.0 (1)
Alanine	1.2 (1)	1.0 (1)	1.1 (1)
Valine	1.0 (1)	1.0 (1)	0.9 (1)
Methionine	0.9 (1)	0.4 (0)	0.9 (1)
Leucine	2.2 (2)	2.1 (2)	1.9 (2)
Tyrosine	2.1 (2)	1.8 (2)	2.0 (2)
Phenylalanine	2.0 (2)	2.0 (2)	2.0 (2)
Lysine	1.0 (1)	1.0 (1)	1.1 (1)
Histidine	1.1 (1)	0.9 (1)	1.1 (1)
Arginine	1.9 (2)	1.8 (2)	1.9 (2)
5-methylmethionine	<0..01 (0)[b]	0.7 (1)	0.1 (0)
Homoserine (lactone)	<0.01 (0)	<0.01 (0)	0.1 (0)
Tryptophan	n.d. [c](1)	n.d. (1)	n.d. (1)
Methionine sulfone	<0.01 (0)	<0.01 (0)[d]	<0.01 (0)[e]

a The theoretical value is given in parenthesis; b detection limit; c n.d. not determined;
d after performic acid treatment; c after treatment with CNBr followed by reduction with dithioerythritol

hundred µl aliquots of these dilutions were mixed with portions of the freshly prepared hepatocyte suspension in polystyrene tubes (5–10 mg dry weight per tube) to a final volume of 1 ml. The tubes were then filled with oxygen and capped. Each concentration of glucagon (or derivatives) was tested in duplicate. The tubes were incubated in a Dubnoff shaker at 37 °C for 30 minutes. Incubation was stopped by addition of 0.5 ml 1.2 M $HClO_4$.

Glucose determination

After centrifugation of the incubation tubes, glucose was determined in the supernatant by an enzymatic method (*Bergmeyer* and *Bernt* 1970). Glucose production during incubation was calculated by correcting for the glucose content in the extracts of cells, which are mixed with $HClO_4$ at t = 0.

For each experiment, glucose production in the individual incubations was expressed as percent of the glucose production in the incubations with 10^{-6} M native glucagon.

Results and Discussion

Antisera

Out of 18 rabbits immunized with S-methylglucagon, two produced antibodies with high affinity, while with methionine sulfoxide derivative only antisera with lower affinity were obtained. Apparently modification of glucagon, other than coupling to larger molecules, can make the molecule immunogenic. Figure 2 shows standard displacement curves of antiserum K 1711 with pancreatic glucagon and with gut-GL1 1. Cross reaction of this antiserum with gut GL1-1 is less than 1%. The sensitivity for glucagon in buffer solution is better than 10 pg/tube.

We have no unequivocal explanation for the high cross reactivity of antiserum K 1711 with native

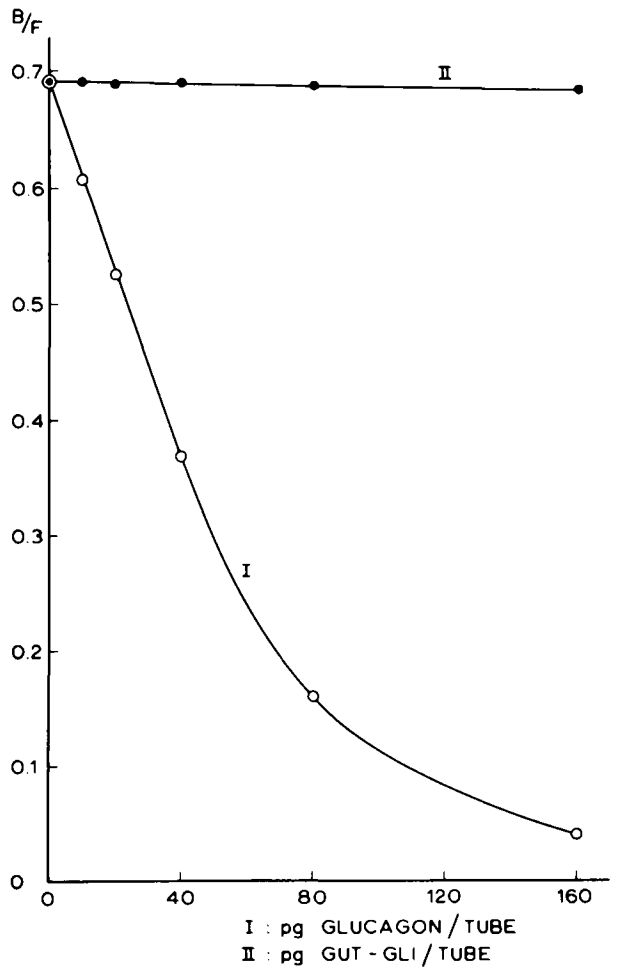

Fig. 2 Standard displacement curves of pancreatic glucagon (I) and gut GL1-1 (II) with antiserum K 1711

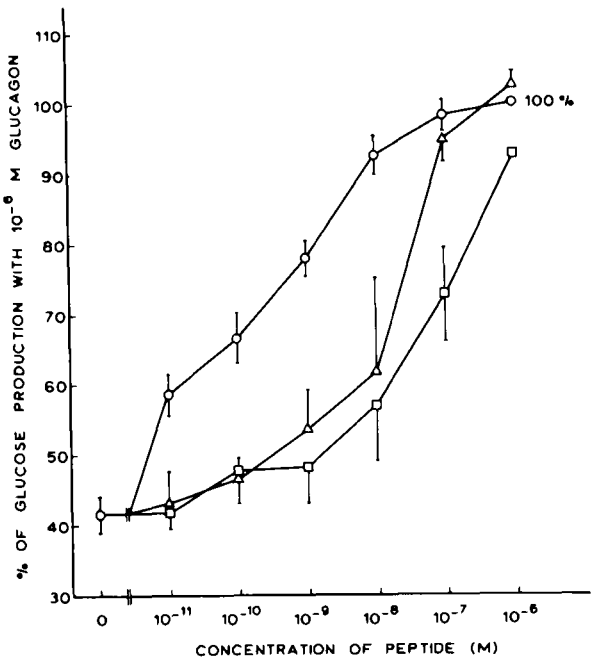

Fig. 3 Effect of glucagon (o—o), glucagon sulfoxide (Δ——Δ), or S-methyl-glucagon (□——□), on glucose production by isolated hepatocytes of fed rats. Mean ± S.D. of three separate experiments

glucagon. Probably the antibodies are not directed against the modified methionine residue itself, but against an adjacent region in the C-terminal part of the glucagon molecule. Preliminary results using K 1711 for radioimmunoassay in human serum showed fasting pancreatic glucagon levels of 20–40 pg/ml.

Bioactivity

Results are shown in Figure 3. The maximal level of stimulation is about 2.5 times the basal glucose production and is equal for glucagon and the sulfoxide; the S-methylderivative also seems to be able to reach this level. While glucagon stimulates glucose production already significantly at 10^{-11} M, the lowest concentration of the sulfoxide that causes a clear effect is 10^{-9} M and of the S-methyl derivative 10^{-8} M.

Our findings are in line with the results of *Rothgeb, Jones, Hayes* and *Gurd* (1977), who observed a 500 fold reduced activity upon methylation of the methionine-27 residue of glucagon (prepared in an almost identical way) on rat liver membrane adenylate cyclase. We find a similar reduction of the half-maximally stimulating concentration of S-methylglucagon as compared to native glucagon. However, the dose-response curves for the stimulation of glucose production, as reported here, are shifted to the left as compared to the stimulation of the adenyl cyclase, shown by *Rothgeb* et al. This may reflect the amplification of the signal between adenylat cyclase stimulation and glycogen phosphorylase activation.

In the only study on the activity of the sulfoxide derivative known to us, it was found that oxidation of methionine-27 to the sulfoxide does not affect lipolytic activity (*Felts* et al. 1970), but concentrations were not given.

The described modifications of the methionine residue of glucagon apparently do not interfere with its maximal glucose mobilizing activity. The dose-response curves for the glucagon derivatives are consistent with a decrease in affinity of those molecules for the glucagon receptor.

Theoretically the glucose mobilizing activity of the glucagon derivatives could be due to contamination with traces of native glucagon. For S-methylglucagon this seems very unlikely in view of its clear separation from native glucagon on the ion-exchange column (see Fig. 1) and because repeated purification did not result in a further reduction of activity.

The sulfoxide was prepared by the addition of 10 equivalents of oxidising agent, although complete oxidation (as judged by amino acid analysis) was equally well achieved by one equivalent (not shown). Contamination with unreacted glucagon should have differentiated these two preparations, yet their biological activities were indistinguishable, indicating the absence of any contamination in either case.

From studies with synthetic glucagon fragments, *Assan* and *Slusher* (1972) inferred that the (24–27) segment is necessary for glucose mobilising function. Our studies are in agreement with this conclusion and further point to a crucial role of the methionine residue in this segment.

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