SYNTHESIS AND ISOLATION OF A GLUCAGON ANTAGONIST

Marvin D. BREGMAN and Victor J. HRUBY
Department of Chemistry, University of Arizona Tucson, AZ 85721, USA

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1. Introduction

The glucagon binding to hepatocytes results in an immediate increase in intracellular cyclic AMP [1], which is followed by an increase in glycogenolysis and gluconeogenesis [2]. It has been suggested that glucagon may be involved in the pathogenesis of diabetes mellitus [3]. A specific glucagon antagonist could prove useful in determining what relationship the hormone has with this disease state. Previous investigators [4] have provided evidence that '(des-His^1)-glucagon', synthesized by the one-step Edman procedure is a competitive inhibitor of glucagon action on liver plasma membrane adenylate cyclase. A highly purified (des-His^1)-glucagon was synthesized by the solid state Edman procedure, which insures the integrity of the ϵ-amino group, and was shown [5] to be a partial agonist. Therefore, the identity of the antagonist remains in doubt. In view of the potential usefulness of this inhibitor we decided to reinvestigate the reaction of phenylisothiocyanate with glucagon. Here we describe the purification and identity of the glucagon antagonist synthesized by the one-step Edman degradation and report on some of its properties which explain the previous differences.

2. Materials and methods

2.1. *Materials*

Crystalline glucagon was obtained from Elanco Co. DEAE A-25 and Sephadex G-10 were purchased from Pharmacia Fine Chemicals. ATP, GTP, cyclic AMP, carboxypeptidase A, dithiothreitol, phosphocreatine and phosphokinase were obtained from Sigma. Aminopeptidase M was from Rohm and Haas. [α-^{32}P]-ATP and cyclic [^{3}H]AMP were purchased from New England Nuclear. All other chemicals were reagent grade.

Abbreviations: des-His^1, des-Histidine; PTC, phenylthiocarbomoyl

2.2. *Synthesis of (des-His^1)(N$^\epsilon$-phenylthiocarbamoyl)-glucagon*

Glucagon (40 mg) was dissolved in 3 ml pyridine/H_2O/triethylamine (70:30:0.01) and adjusted to pH 10.4 with triethylamine. Nitrogen was bubbled through the solution, then phenylisothiocyanate (150 μl) was added. The mixture was incubated at 37°C for 2 h, then extracted with benzene until the benzene layer was clear. The aqueous layer was taken to dryness in vacuo. Trifluoroacetic acid (10 ml) containing dithiothreitol (3 mg/ml) was added and the sample was sealed under nitrogen for 10 min at 50°C. The sample was again taken to dryness in vacuo. The resultant product was then chromatographed on a Sephadex G-10 column (0.8 × 31 cm) which was eluted with pyridine/H_2O (1:2). After lyopholization, the crude product was dissolved in Tris–urea buffer (pH 7.7) and chromatographed on DEAE–Sephadex by methods similar to those in [6]. The purified title compound was isolated and lyophilized to give a white powder (25 mg).

2.3. *Amino acid analysis*

The products were analyzed on a Beckman 120 C amino acid analyzer following either acid hydrolysis by 6 N HCl for 22 h at 110°C or by proteolysis. The peptide sample (~1 mg/ml) was incubated with aminopeptidase M (1%, w/w) for 24 h at 30°C in 20 mM $NaHCO_3$ followed by carboxypeptidase A

(2%, w/w) for 24 h. The enzyme-digested sample was added directly to the amino acid analyzer.

2.4. *Liver plasma membrane adenylate cyclase assay*

Liver plasma membranes from rat livers were prepared by a modification of the procedure [7] as in [8]. Except for the addition of GTP (10 μM) incubation conditions were as in [9]. Cyclic AMP was determined by the method in [10]. Protein was determined by the Lowry method [11] using bovine albumin as a standard.

2.5. *Absorption measurements*

Ultraviolet and visible absorption measurements were made on a Gilford 240 spectrophotometer.

3. Results

3.1. *Purification and identification of glucagon derivatives synthesized by the Edman degradation reaction*

Analysis of the products derived from the reaction of glucagon with phenylisothiocyanate by DEAE–Sephadex chromatography is shown in fig.1. Acid hydrolysates of the material in peak A (fig.1) yielded an amino acid analysis consistent with (des-His^1)-glucagon (table 1). Enzyme digests of this peptide material in peak A yielded the same analysis, showing that the ϵ-amino group of Lys-12 was not modified. This was as expected since work with DEAE–Sephadex A-25 has shown that glucagon was not held by the column unless there was a decrease in net positive charge, such as neutralization of the ϵ-amino group [6]. As expected, amino acid analysis of the material in peak B following peptidase treatment revealed the loss of lysine (table 1). Acid or base hydrolysis of (des-His^1) (N^{ϵ}-PTC)-glucagon led to regeneration of the lost lysine (not shown). This was as expected since in his earliest work Edman reported [12] that the N^{ϵ}-PTC group of lysine was removed by base hydrolysis. It is also known that in acid hydrolysis regeneration of free lysine occurs [13]. In this regard, the work in [4] relied solely on acid hydrolysates for their analysis thus explaining why they detected none of the N^{ϵ}-PTC-lysine which quite likely was present in their preparation. We further confirmed the presence of the phenylthiocarbomoyl moiety in our purified (des-His^1) (N^{ϵ}-PTC)-glucagon by absorption measurements. The ratio (A_{260}/A_{280}) was 0.72 for glucagon and 1.42 for (des-His^1) (N^{ϵ}-PTC)-glucagon at pH 10.0. This increase in the ratio is the result of the PTC moiety which contributes very substantially to A_{260} [14]. The distribution of material between peak A and peak B was directly dependent on the pH of the pyridine/water solution used in the synthesis. Formation of (des-His^1) (N^{ϵ}-PTC)-glucagon (peak B) was favored at the higher pH values, with 90% of the peptide material being found in peak B at pH 10.4. Lowering the pH increases the amount of (des-His^1)-glucagon (peak A) formed. For proper preparation of the antagonist (des-His^1)

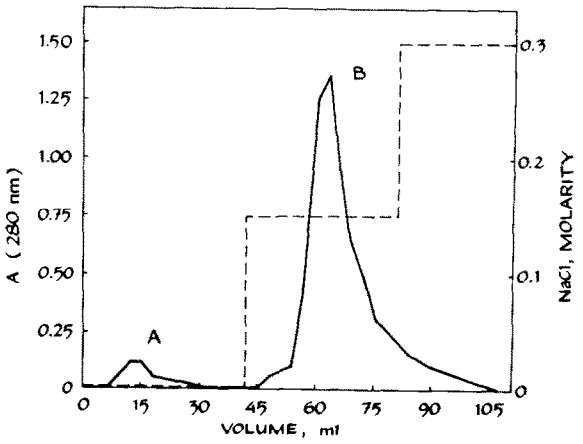

Fig.1. DEAE–Sephadex chromatography of the products derived from the reaction of glucagon with phenylisothiocyanate (——). Tris buffer, 0.01 M, in 7 M urea (pH 7.7) was used as eluent solvent with a discontinuous NaCl gradient (- - - -).

Table 1
Amino acid analysis – basic amino acids

Amino acid	Glucagon calc.	Peak A acid hydrolysis	Enzymatic digests	Peak B enzymatic digest
Tryptophan	1.0	ud[a]	0.88	0.92
Lysine	1.0	0.98	0.95	0.02
Histidine	1.0	0.01	0.01	0.01
Arginine	2.0	1.97	1.99	1.94

[a] Glucagon contains 1 residue of tryptophan which is lost on acid hydrolysis

(N^{ϵ}-PTC)-glucagon it is critical, therefore, that the pH be carefully controlled during the synthesis.

3.2. *Effect of glucagon analogues on liver adenylate cyclase*

(des-His^1)-glucagon (peak A) is a partial agonist, stimulating liver adenylate cyclase activity to 56% of the maximum level obtained with glucagon alone (fig.2). This partial agonism is very similar to that reported for (des-His^1)-glucagon prepared by the solid-phase Edman procedure [5]. On the other hand (des-His^1) (N^{ϵ}-PTC)-glucagon had essentially no effect on liver adenylate cyclase activity (fig.2). Maximal activity observed was 5% at 1 μM, and this probably reflects a slight (1–3%) contamination of (des-His^1)-glucagon. This was further affirmed by the competitive inhibitory action of (des-His^1) (N^{ϵ}-PTC)-glucagon on glucagon stimulation liver adenylate cyclase activity (fig.2). (des-His^1) (N^{ϵ}-PTC)-glucagon (1.5 μM) caused a 26-fold increase in the glucagon concentration required for 50% activation (K_a). Assuming the receptor occupancy is directly proportional to the adenylate cyclase activity in these studies, the antagonist, (des-His^1) (N^{ϵ}-PTC)-glucagon, would have 1/18th the affinity of glucagon for the receptor site.

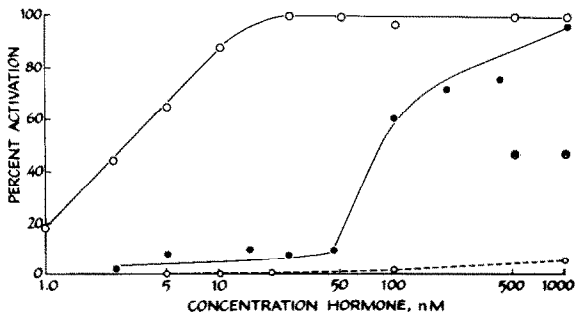

Fig.2. Effect of (des-His^1) (N^{ϵ}-PTC)-glucagon on the glucagon stimulation of liver adenylate cyclase activity. Assay conditions are in section 2. The protein content per assay was 30 μg. The stimulatory effect of glucagon (–○–), (des-His^1) (N^{ϵ}-PTC)-glucagon (- - -), (des-His^1)-glucagon (×) and glucagon containing (des-His^1) (N^{ϵ}-PTC)-glucagon, 1.5 μM, (–●–) on the adenylate cyclase activity is expressed as % activation over basal. 0% activation represents the basal activity (270 pmol/10 min/mg protein) and 100% is the maximum activity observed with glucagon (2200 pmol/10 min/mg protein). The results are the mean of 3 determinations each from triplicate experiments.

4. Discussion

This report clearly shows that the glucagon antagonist synthesized by the Edman degradation of glucagon is (des-His^1) (N^{ϵ}-PTC)-glucagon. However, (des-His^1)-glucagon is a partial agonist ([5] fig.2) as was reported. These results indicate that introduction of the PTC moiety into the ϵ-amino position of glucagon is primarily responsible for generating an antagonistic form of the hormone. Other studies have shown that incorporation of a hydrophobic moiety into the ϵ-amino position results in a loss of biological activity [9,15,16]. Physical studies on these derivatives [6,16] suggest that their molecular conformation is much more complex than the flexible random coil form of monomeric glucagon [17–19]. Guanidation of the lysine position does not result in an inactive derivative [20], which suggests that neutralization of the ϵ-amino group may be an important factor contributing to the new conformation. Additional work will be needed to further evaluate the importance of these various factors and to obtain a better understanding of the conformational properties of these compounds. In addition, this antagonist may prove useful in evaluating the actions of glucagon in in vitro and in vivo systems. Work is currently in progress in our laboratory in these areas, and in the synthesis of a variety of related glucagon analogues.

Acknowledgements

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