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SUCCINYLATED GLUCAGON: PREPARATION, SOME PHYSICAL PROPERTIES AND HORMONAL ACTION

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Summary

A succinylated derivative of glucagon has been prepared and its purity is established. It is much more soluble than glucagon at neutral pH and has a pI of about 3.3. Proton magnetic resonance studies clearly show its existence as a random coil. Its adenyl cyclase stimulatory activity is about 40—50% of that of glucagon.

Introduction

Glucagon is a 29-amino acid polypeptide hormone of known sequence[1,2] and three-dimensional structure[3]. In dilute aqueous solutions, it exists as an equilibrium population of conformers and molecular units[4], especially monomer and trimer[5]. Upon self-association[4] and binding with lipid micelles[6] the structure becomes more ordered. Glucagon has several easily modifiable functional groups and studies have been made on the physical and biological properties of the chemically modified glucagon derivatives[7–13]. The α-amino of the N-terminal histidine and ε-amino of lysine have been acetylated, carbamylated and trinitrophenylated.[10,11,13,14]

In opposition to the action of insulin, glucagon raises the plasma glucose levels. Upon binding to a receptor site on the plasma membrane, the coupled adenyl cyclase activity is raised resulting in an increase of cyclic AMP levels. This second messenger then proceeds to activate a series of enzymes leading to the formation of glucose. In diabetic subjects having high plasma glucose levels, not only is the insulin activity low, but the plasma glucagon is also high compared with normal subjects. Diabetes could thus be considered a disease arising from the abnormal level of *both* glucagon and insulin in the plasma. Recent studies on cellular and subcellular systems using modified glucagons are made with the aim of finding useful derivatives which may help in the elucidation of the hormone structure in solution, its mode of action, the nature of the hormone-receptor interactions, its metabolism and may eventually help in the therapy of diabetes. A useful derivative may have an activity which is different from that of glucagon or may even be inhibitory to the glucagon action.

In this communication, we describe the preparation of succinylated glucagon, some of its physical properties and its effects on rat liver membrane adenyl cyclase activity.

Materials and Methods

Glucagon, creatine kinase, phosphocreatine, adenosinetriphosphate, ^{3}H-cyclic AMP, anhydrous theophylline crystals, 2,5-diphenyloxazole, p-bis-(2-(5-phenyloxazolyl)-benzene) and 2,4,6-trinitrobenzene sulfonic acid (TNBS) were all purchased from Sigma Chemical Company, St. Louis, Mo, USA. Succinic anhydride was from May and Baker Ltd., Dagenham, England. Acrylamide, *N,N*-methylene bisacrylamide and *N,N,N',N'*-tetramethyl ethylene diamine were obtained from Eastman Kodak Company, Rochester, N.Y., USA. Carrier Ampholytes were from LKB and Sephadex G-10 from Pharmacia Fine Chemicals, Uppsala, Sweden. Guanidine hydrochloride (British Drug Houses Ltd., U.K.) was the spectroscopic-grade material exhibiting no absorption at 230nm at 6 M concentration. Protein kinase from rabbit muscle was a gift from Dr. A. Haesungcharern. Rats were originally from Rolfsmeyer Farm, Madison, Wisconsin, USA.

Succinylation of glucagon followed a method modified from that of Klotz[15]. 100-fold molar excess of crushed succinic anhydride was slowly added to a suspension of 20 mg glucagon in 5 ml of water maintained at pH 6–7 throughout. Glucagon completely dissolved after about 15% of the anhydride had been added. Upon completion of addition the solution was applied to a Sephadex G-10 column (15×90 cm) equilibrated with ethanol: water (2:1, v:v) and the glucagon derivative was eluted by the same solvent system. Fractions were collected at a rate of 10 ml/hour and monitored at 278 nm. Pooling of fractions with O.D. above 0.1 followed by lyophilization produced a salt-free product. This was assayed for free amino groups at 420 nm by the TNBS method of Fields[16] using glucagon and lysine as standards. Acrylamide gel electrophoresis was performed at pH 9.1, in 6 cm gels, and a current of 4 mA/tube as described by Davis[17]. Coomassie Blue was used for staining. Isoelectric focusing was adapted from the procedure recommended by Wrigley[18] using 10 cm gels and a current of 1.5 mA/tube. Glucagon concentration was determined spectrophotometrically using an $E^{1\%}_{1\,cm}$ at 278 nm of 2.38[4]. Proton magnetic resonance spectra were recorded on a 90 MHz Bruker Spectrospin instrument with Pulsed-Fourier-Transform accessory. Each spectrum was the resultant of 2046 accumulated scans. Exchangeable protons in the solution were all replaced by deuterium by repeated dissolutions in D_2O and lyophilizations. Membrane protein was estimated by the method of Lowry *et al.*[19]. Rat liver plasma membrane preparation was modified from that of Neville[20] and the adenyl cyclase assay was adapted from that of Pohl *et al.*[21]. Cyclic AMP determination followed, with minor modifications, the protein kinase method of Gilman[22]. Scintillation counting was done in a Packard Scintillation Counter.

Results and Discussion

Succinylated glucagon was eluted from the column at the void volume (66 ml) giving a single symmetrical peak. Pooling of fractions and lyophilization gave a colourless solid with a yield of over 95%. The derivative was found to have less than two percent free amino groups by the TNBS assay. Disc-gel electrophoresis gave one band which traveled about twice as fast as the glucagon major band.

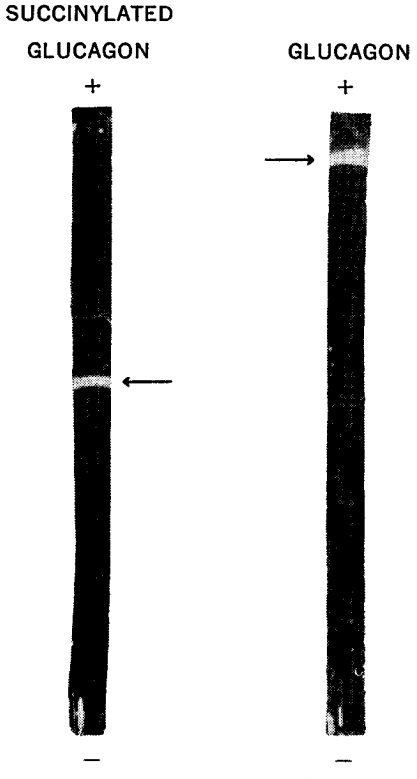

Fig. 1. Disc-gel electrofocusing of succinylated glucagon (left) and glucagon (right). Precipitation bands are indicated by arrows.

Electrofocusing showed a major precipitation band with an estimated pI of~3.3 for succinylated glucagon vs 6.2 for glucagon (Fig. 1). Thus the succinylated glucagon was virtually pure. Since there are two free amino groups per glucagon, probably the molecule was disuccinylated. Modification of other groups, e.g., hydroxyls of the three serines, was unlikely under the conditions used[15]. The UV spectrum of the derivative was nearly identical with that of glucagon and showed similar pH dependence. Unlike glucagon, it was rather insoluble from pH 1 to 4, but became highly soluble (up to 20 mg/ml) from pH 5 upwards. Glucagon is relatively insoluble from pH 4 to 9.5 (up to 0.35 mg/ml only) but is more soluble at the two pH extremes (up to 10 mg/ml at pH 10.2). All this is consistent with the complete substitution of the two positively charged amino groups by two negatively charged carboxylic groups of the succinylated side chains. Presumably, monosubstituted species are also highly soluble as witnessed by the total dissolution of the glucagon suspension upon addition of a small percentage of succinic anhydride.

The conformation of succinylated glucagon in solution was previously studied by circular dichroism in the presence and absence of guanidine hydrochloride[14]. These studies showed that succinylated glucagon was probably a random coil. But the combination of circular dichroism and proton magnetic resonance spectra clearly show that it is undisputably so. Fig. 2 shows the proton magnetic resonance spectrum of succinylated glucagon at 20 mg/ml in 6 M guanidine hydrochloride at pH 10.2. The peaks are sharp and well-resolved for a polypeptide of this size. The absence of a large HDO peak indicates a virtual absence of H_2O. The spectrum fits very well with that obtained from the theoretical contributions of the individual amino acid side chains of the hormone. Since the freedom of motion of amino acid side chains in a random coil is believed to be comparable to that of free amino acid side groups[23], this good fit leads to a definite assignment of the succinylated glucagon conformation in 6 M guanidine hydrochloride: a random coil. Succinylated glucagon at 10 mg/ml and 20 mg/ml, in the absence of guanidine chloride, also showed exactly the same proton magnetic resonance features of the amino acid side chains as those in Fig. 2. Thus succinylated glucagon is a true random coil in solution without the presence of "denaturants" such as guanidine hydrochloride and urea[24]. The spectrum can be contrasted with that of glucagon which shows broader peaks even in the presence of guanidine hydrochloride[4]. Thus it is suggested that succinylated glucagon should be seriously considered as a good model for a natural polypeptide random coil.

The basal activity of our rat liver membrane preparation was 3.3 nanomoles of cyclic AMP/10 minutes per 1 mg membrane protein. This value is relatively high compared with the activity found by some workers[7,25]. One washing did not bring the value down significantly. Twofold stimulation by 1 μM of glucagon was observed. Succinylated glucagon was less active than glucagon and found to have about 40–50% of glucagon activity (see Table I). This reduced activity is comparable to findings with other derivatives having substituted amino groups[11,13]. Apparenty, the amino groups are essential for full activity. Since succinylated glucagon exists as a random-coil monomer which does not aggregate in solution, therefore, its hormonal

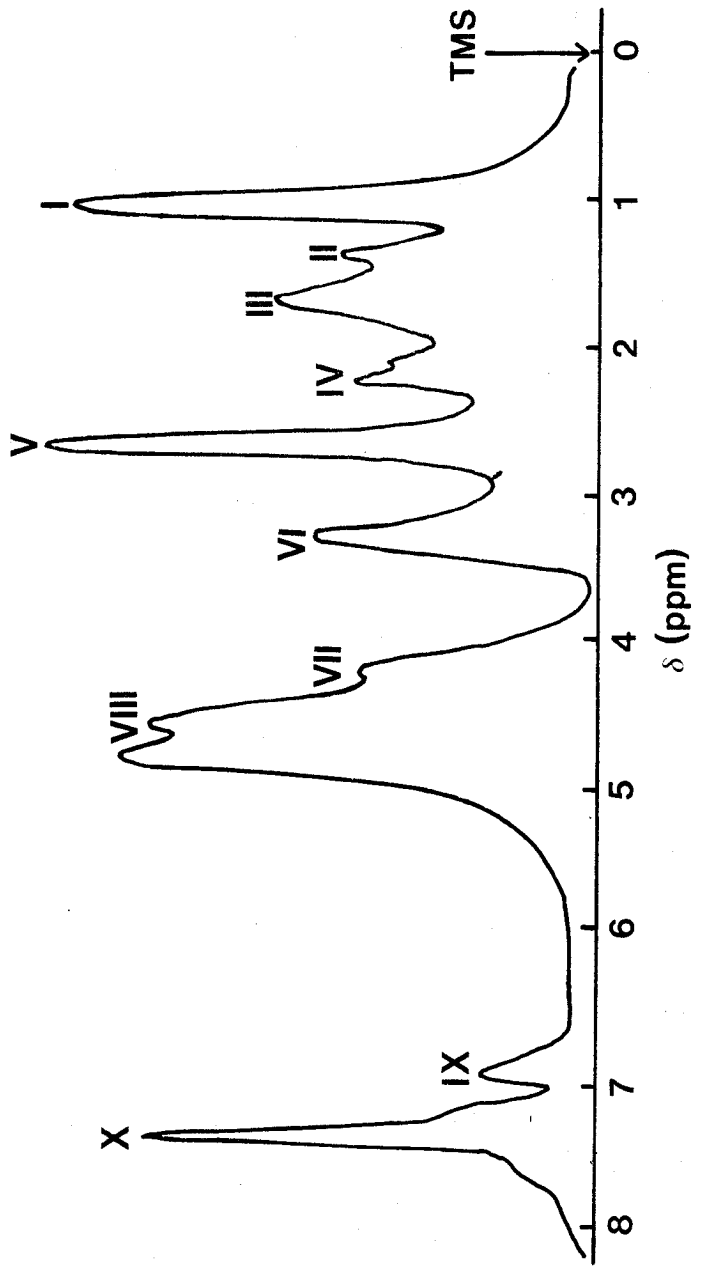

Fig. 2. Pulse Fourier Transform proton magnetic resonance spectrum of succinylated glucagon (20 mg/ml, pH 10.2) in the presence of 6 M guanidine hydrochloride in D_2O. Peak assignments: I, $-CH_3$ of leu and val; II and III, $-CH_3$ of ala and thr; IV, $-CH_3$ of met; V and VI, $-CH_2$ of ser, lys, arg, asp and glu; VII, α–CH of gly; VIII, α–CH of other residues; IX, aromatic H of tyr and try; X, aromatic H of phe, tyr, try and his.

TABLE I: ADENYL CYCLASE ACTIVITY OF RAT LIVER MEMBRANE[a]

System	Hormonal stimulation[b] nmol of cAMP/10 min/mg membrane protein per μM hormone
Membrane + glucagon	5.10 $\pm$ 0.54
Membrane + succinylated glucagon	1.82 $\pm$ 0.05

[a]Membrane basal activity = 3.28 $\pm$ 0.19 nmol cAMP/10 min/mg membrane protein.

[b]Hormonal stimulation is the adenyl cyclase activity of the membrane preparation in the presence of 1 μM of hormone minus basal activity.

activity, albeit reduced, indicates that both it and glucagon may also be active as a monomer and that the active conformation may be induced upon binding to the plasma membrane. To establish the usefulness of the succinylated glucagon, naturally, needs further experiments including trials in experimental animals.

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บทคัดย่อ

อนุพันธ์กลูคากอน disuccinylated glucagon เตรียมได้จากการทำปฏิกิริยาระหว่างกลูคากอนกับ succinic anhydride พิสูจน์ได้ว่าอนุพันธ์นี้บริสุทธิ์ เมื่อเทียบกับกลูคากอน สารที่เตรียมได้มีความสามารถในการละลายน้ำได้ดีกว่ามากในช่วง pH กลาง ๆ และมีค่า pI เท่ากับ 3.3 ผลการทดลองโดยใช้ nuclear magnetic resonance แสดงอย่างแน่ชัดว่ามันอยู่ในสภาพ random coil ความสามารถในการกระตุ้นระบบ adenyl cyclase ของเยื่อหุ้มเซลล์ตับเป็นประมาณ 40–50 % ของกลูคากอน