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Proteolysis of Glucagon Bound to Dimyristoylphosphatidylcholine Vesicle

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Glucagon was found to interact with DMPC vesicles electrostatically and hydrophobically. It appears that glucagon bound irreversibly to the vesicles through hydrophobic interaction was partially protected from the proteolysis by trypsin. Out of three possible sites, only the peptide bond preceded by Arg-18 was cleaved by a prolonged trypsintreatment. α-chymotrysin did not affect the vesicle-bound glucagon. Based on these observations, possible structure of irreversibly bound glucagon on the vesicle surface is discussed.

Introduction

Many mammalian glucagons have an identical amino acid sequence consisted of 29 amino acid residues as shown below. This protein,

His – Ser – Glu – Gly – Thr – Phe – Thr – Ser – Asp – Tyr – Ser – Lys – Tyr – Leu – Asp – Ser – Arg – Arg – Ala – Glu – Asp – Phe – Val – Glu – Trp – Leu – Met – Asn – Thr

(5, 10, 15, 20, 25)

like many other peptide hormones, have a high potential of forming amphiphilic helical structures[1,2]. The amphiphilicity of parts of the sequence is a common feature among peptide hormones, enabling them to form stable complexes with lipid vesicles. The capacity of assuming an amphiphilic secondary structure accompanying hormone-membrane interaction may have physiological roles as suggested by Tayler and Kaiser[2]. For example, an amphiphilic α-helix or a β-strand may interact with a complementary site on a receptor protein in a manner analogous to its interaction with phospholipid[3]. A second possibility is that amphiphilic secondary structures may facilitate interaction between peptide hormones and receptors through two dimensional diffusion on cell surface[4,5]. Also, the peptide hormone binding to membrane surface may either protect the peptides from attack by soluble proteolytic enzymes or lead to more rapid and specific breakdown by membrane-associated proteases[6]. Pasta *et al.*[7] observed that micelles of various surfactants prolonged the hydrolysis of glucagon by trypsin and α-chymotrypsin. However, they obtained an identical set of products from the digestion of glucagon with or without the presence of micelles. Here we have focused on the interaction of the glucagon with dimyristoylphosphatidylcholine (DMPC) vesicles and the effect of the vesicles on the mode of proteolysis of the peptide hormone by trypsin and α-chymotrypsin. It was found that a certain peptide bonds are protected from the proteolysis by trypsin when glucagon is bound to vesicles. The vesicle-bound glucagon was completely protected from α-chymotrypsin.

Experimental

Materials. Glucagon (extracted from mixture of bovine and porcine pancreas), trypsin (from bovine pancreas, tre-

ated with TPCK (tosyl-L-phenylalanin chloromethyl ketone)), α-chymotrypsin (from bovine pancreas, treated with TLCK), DMPC and phenylmethylsulfonylfluoride (PMSF), were purchased from Sigma Chemicals. HPLC solvents were purchased from Baker. Polyamide plates were from Pierce. All other chemicals were obtained as the highest grade available. A Waters Associated liquid chromatography equipped with a Waters 740 data module and Z-module/radial-pak μ-Bondapak C_{18} column (0.8 × 10 cm) was used for analyzing the digestion products.

Preparation of Glucagon Solution. Glucagon was dissolved in 0.2 M ammonium bicarbonate buffer (pH 7.4)[8]. The glucagon solution was spun (15,000 g, 10 min) with an Eppendorf centrifuge and the clear supernatant obtained was used for subsequent experiments. The concentration of glucagon was determined spectrophotometrically at the wavelength of 278 nm using the absorption coefficient of 2.38[9].

Preparation of Phospholipid Vesicles. Homogeneous large unilamellar vesicles(LUV) of DMPC were prepared by the reverse-phase evaporation (REV) method[10]. Phospholipid concentration was determined according to the method of Vaskowsky *et al.*[11].

Binding of Glucagon to DMPC Vesicles. Centrifugation method was used to quantitatively determine the binding of glucagon to vesicles at the ionic strength of 0.2 or 0.5. After equilibrium was reached (4 hours), the glucagon-vesicle complexes were precipitated by centrifugation (Beckman TL-100 rotor, 100,000 g, 90 min), and the supernatant was analyzed for protein by the modified Lowry method[12]. From the concentration difference between this and the original glucagon solution, the amount of bound glucagon was estimated. The interation of glucagon with vesicles was also studied with fluorescence spectroscopic method. The intrinsic fluorescence of glucagon was recorded with a Jasco FP-770 spectrofluorometer using 1 cm pathlength cuvettes at 25 °C. Fluorescence emission from 280 nm to 450 nm was detected after exciting the sample at 280 nm. The intrinsic fluorescence of glucagon in 0.2 M ammonium bicarbonate buffer (pH 7.4) and in the DMPC vesicle system was compared. The lipid/protein (L/P) ratio was 100.

Proteolysis. Proteolysis of glucagon by trypsin and α-chymotrypsin was carried out in 0.2 M ammonium bicarbonate buffer (pH 7.4) at 25 °C (protein/enzyme mole ratio = 10). Duration of proteolysis ranged from 5 min to 24 hours. The mode of proteolysis of glucagon-vesicle complexes under various condition was compared with that of free glucagon. The membrane-bound glucagon was separated from free glucagon by centrifugation. Two different ionic strengths (I = 0.2 and 0.5) were used in order to check possible effect of electrostatic interaction between enzyme and glucagon-vesicle complex on the hydrolysis. The digestion was stopped by the addition of fresh PMSF solution (52 mg/ml in ethanol) to 3 mM final concentration, followed by incubation at 37 °C, 3 min.

Separation of Digested Fragments by HPLC. 3 volumes of chloroform/methanol (2:1) mixture were added to 1 volume of suspension of the glucagon-vesicle complex after the proteolytic digestion and the interphasial fluffs were separated. The digested fragments were recovered after evaporating solvents by passing N_2 gas and dissolved in elution buffer. The solution was analyzed by HPLC using a Z-module/radial-pak μ-Bondapak C_{18} column (0.8×10 cm). Elution was performed with a gradient of acetonitrile concentration from 0% to 60% in 50 mM sodium phosphate buffer (pH 3). The flow rate was 2.5 ml/min and the eluent was measured at 280 nm. The retention times were calculated with a Waters 740 data module.

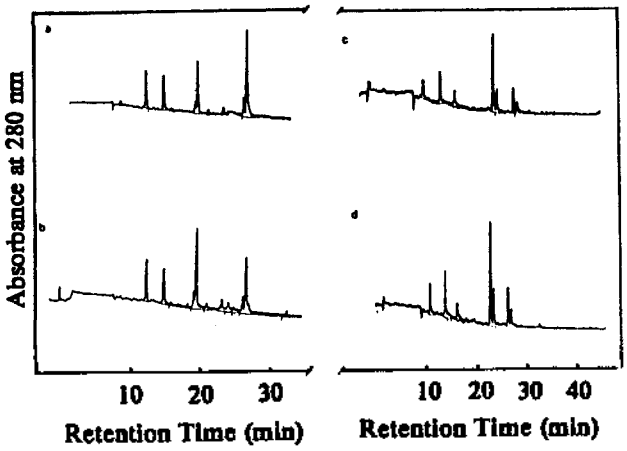

Figure 1. HPLC profile of glucagon fragments obtained by tryptic digestioin of free glucagon at 25 °C. a: 5 min of proteolysis in 0.2 M buffer. A similar pattern was obtained after 30 min of digestion. b: 1 hour of proteolysis in 0.2 M buffer. c: 10 hours of proteolysis in 0.2 M buffer. d: 10 hour of proteolysis in 0.5 M buffer.

Identification of the Fragments within the Sequence. (1) *Determination of N-terminal amino acid residues.* -The fragments obtained from proteolytic digestion of glucagon-vesicle complex were extracted and separated by HPLC as described above. The amino-terminal residues of these fragments were dansylated. After digestion with 6 N HCl, the amino acid residues were separated on a TLC plate[13]. (2) *Analysis of amino acid composition of the fragments.* – The same fragments which were used for N-terminal determination were dried by flushing with nitrogen gas and 50 μl of 6 N HCl was added. The fragment was hydrolyzed at 105 °C for 20 hours. 90 μl of 0.2 M sodium bicarbonate buffer (pH 8.5) was added and dried. 40 μl of dansyl chloride reagent was added and incubated at 37 °C for 30 min in the dark. The dansylated amino acids were analyzed by two-dimensional TLC as described above.

Results and Discussion

Most of the experiments were performed at the L/P ratio of 100 with glucagon concentratin fixed at 0.28 mg/ml. Quantitative analysis of binding was performed using centrifugation method. 40.5% of the protein present was bound at ionic strength of 0.2 and 27.4% at ionic strength of 0.5. These results suggest that there are two binding modes, one due to electrostatic force and the other by hydrophobic interaction. The latter represents the irreversible binding portion of total binding. The involvement of hydrophobic interaction was corroborated by an appreciable influence of the vesicles on the intrinsic fluorescence of glucagon. There was a blue shift of emmision maximum wavelength from 352 nm to 338 nm and a 230% increase in quantum yield when the emission

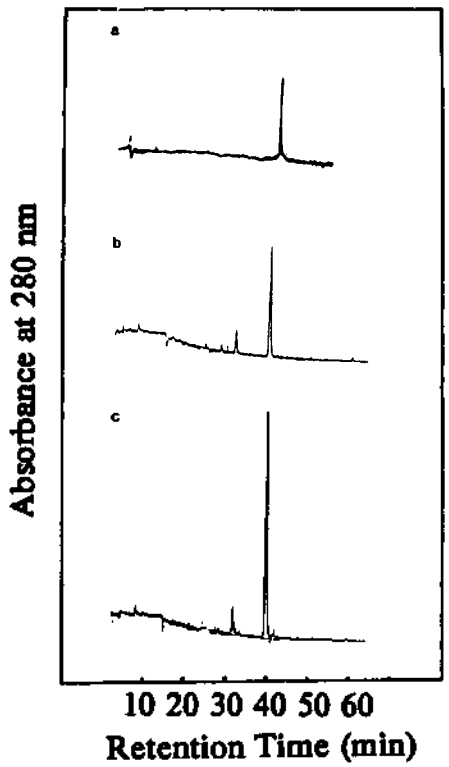

Figure 2. HPLC profile of peptide fragments from glucagon - DMPC vesicle complex hydrolyzed with trypsin. a: 5 min of incubation with trypsin. Only glucagon peak can be seen. b: 1 hour of proteolysis. c: 10 hours of proteolysis. See text for the details.

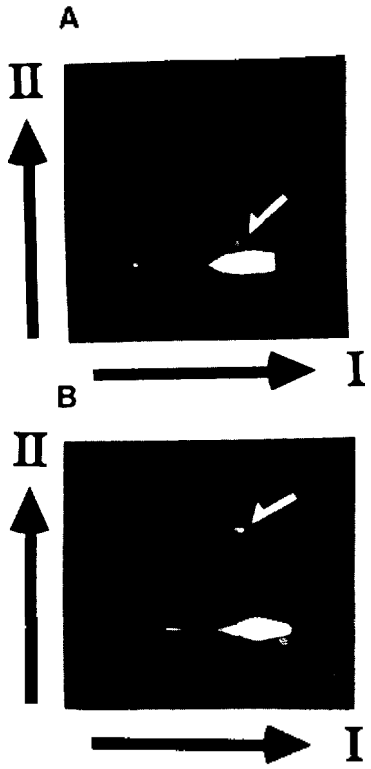

Figure 3. N-terminal amino acid residue of two fragments of glucagon obtained from the tryptic digestion of vesicle-bound glucagon. (A) shows dns-O-Tyr spot (arrow). (B) shows Ala spot (arrow). Identification of the dansylated amino acid residue was done by comparing the R_f values of spots with those of standard compounds.

spectrum from the glucagon, after 4 hours of incubation with vesicles, is compared with that of the glucagon solution without the vesicles. These suggest that the polarity of the environment of Trp-25 decreased when the peptide hormone is bound to vesicles [14]. This, in turn, suggests penetration of the protein into bilayer. Pasta *et al.*[7] obtained similar results when glucagon was allowed to interact with micelles.

The effect of DMPC vesicles on the proteolysis of glucagon was studied by analysing the digestion products with an HPLC. There are one Lys residue and two Arg residues in glucagon and complete tryptic digestion is expected to produce 4 fragments[15]. Figure 1 shows the HPLC profiles of framents obtained by tryptic digestion of free glucagon solution in the absence of vesicle. Althought four peaks are discernible here, the single Arg-18 is not expected to show under the present monitoring method of A_{280} absorption. It is likely, therefore, that incomplete breakdown of the peptide bond between Arg-17 and Arg-18 produced total 4 peaks here [15]. After a prolonged digestion, additional fragments appeared (Figure 1c and 1d). This may be due to the autolysis of trypsin to produce pseudo-trypsin which has an enzymatic property similar to α-chymotrypsin[16]. Figure. 2 presents the HPLC profiles of products of the tryptic digestion of the glucagon/vesicle complex. After 5 min of incubation, only intact glucagon is seen (Figure 2a). This is in contrast to the case of free glucagone and indicates the protection provided by the vesicles. Longer digestion times produced only two peaks (Figure 2b and 2c). Clearly one or more sites are protected from the trypsin. That this is not due to vesicle-modified enzymatic property is shown by an experiment in which the trypsin separated from the vesicles is used for the digestion of free glucagon. The HPLC profile of this was the same as the result shown in Figure 1a. Difference in ionic strength of buffer solutions made little effect on digestion of vesicle-bound glucagon. This shows that electrostatic repulsion between the enzymes and vesicular complex is not the cause of protection from digestion. Therefore, the protection of glucagon which is bound to membrane irreversibly from the soluble proteolytic enzyme must be caused by physical barrier provided by LUV membrane.

The dansylated N-terminal amino acid of one of the two fragments after the tryptic digestion of vesicle-bound glucagon was found to be Ala-19 (Figure 3b). But for the other fragment, only dns-O-Tyr could be identified (Figure 3a). It is likely that the N-terminal amino acid residue of this fragment was His-1, but the bis-dns-His was completely decomposed during acid hydrolysis as is the usual case[17]. This point was tested by determining the combined amino acid composition of the two fragments by dansylation method and the results are shown in Figure 4. The appearance of a dansylated His confirmed that the N-terminal amino acid of one fragment is His-1 since this is the only amino acid residue of this kind in the sequence. In conclusion, only the peptide bond following Arg-18 is broken here.

The partial protection from tryptic digestion may mean that the membrane-bound glucagon is initially protected from soluble proteolytic enzymes present in the body fluid.

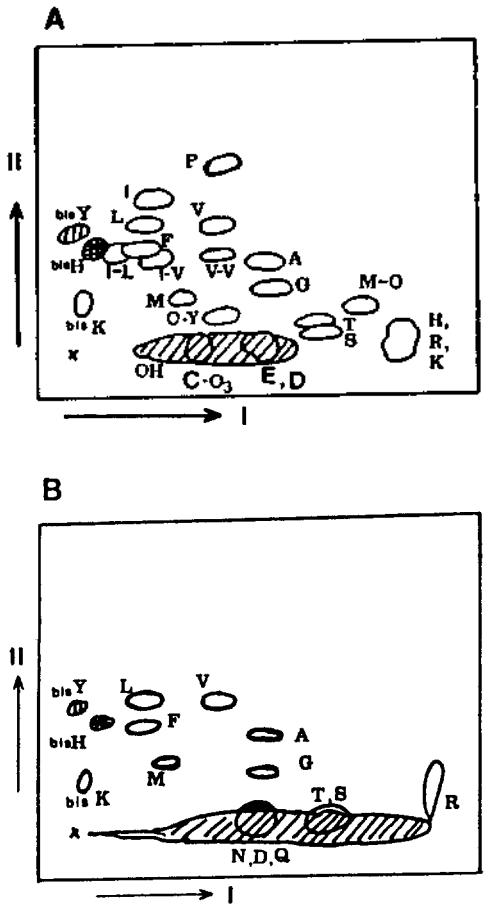

Figure 4. Combined amino acid composition of two fragments of glucagon obtained as in Figure 3. After the tryptic digestion of glucagon/vesicle complex with trypsin, the polypeptide segments were extracted, digested with 6 N HCl and then dansylated prior to the TLC. (A) standard, (B) sample. His spot can be clearly be seen.

However, the specificities of these enzymes are not well defined.

The digestion experiment of glucagon/vesicle complex was repeated with α-chymotrypsin. After the extraction of the protein, it was dansylated and hydrolysed with 6 N HCl followed by TLC. No dansylated amino acid residue was observed. Since the N-terminal residue is not expected to be dansylated, this result is to be expected if α-chymotrypsin did not break any peptide bond of membrane-bound glucagon. If any of the peptide bonds is broken by α-chymotrypsin, one or more of the residues Thr, Val, Ser and Leu should have been dansylated. For the control, the extracted glucagon from the vesicle after α-chymortypsin digestion was hydrolyzed with 6 N HCl and then dansylated. From the 2D-TLC of these products, all the amino acid residues of glucagon were accounted for, indicating that glucagon remained attached to the vesicles after the chymotryptic digestion. A complete digestion of free glucagon by α-chymotrypsin was observed.

These data suggest that the physical barrier of membrane protects glucagon from proteolysis but some residues near the Arg-18 is located outside of membrane. Analysis of the glucagon sequence using the Edmundson helical wheel diagram[18] or helical net diagram indicates potential formationn of two separate amphiphilic domains by residues 5-16 and residues 17-29[2]. However, these will have opposite orientations if they adopt α-helical conformation. With this structure, glucagon would not have much stability because only one domain is attached to the bilayer. According to the NMR studies of glucagon-dodecylphosphocholine micelle complexes by Braun *et al.*[19], the glucagon froms several domains of different structures. Most part of this peptide hormone is associated with the micelle in an extended form while the residues from 1 to 4 are highly mobile, indicating that this N-terminal end juts out into the solution. Within the bound segment, residues 10 to 14 form an α-helix-like turn and residues from 17 to 29 make three irregular α-helix turns. Segments from residues 5 to 10 and from residues 14 to 17 form extended stretches. There are three potential sites in glucagon which can be broken by trypsin; Lys-12, Arg-17 and Arg-18. However, present work has shown that only the peptide bond after Arg-18 is broken. It is also possible Arg-17 was broken as well. It is interesting to note that Lys-12 which is within a helix loop is not broken. Since only amphiphilic helical segments bind tightly to the amphiphilic surface, it is likely that only these are not accessible to trypsin. The Lys-12 within the α-helical loop of 10-14 segment is expected to be oriented away from the bilayer surface. However, previously we observed that basic amino acid residues within an α-helix segment, when bound to the surface of vesicles, are protected from trytic digestion[20-22]. On the other hand, Arg-18 is located at the edge of a helical segment and it may be mobile enough to be accessible to trypsin.

As to the result of proteolysis of membrane-bound glucagon by α-chymotrypsin, most of the aromatic amino residues are located within the helix forming segments. These hydrophobic residues seem to be in contact with hydrophobic interior of lipid bilayer and thus are protected from the enzyme. The only aromatic residue located outside of the putative helices is Phe-6. Although this residue is within an extended stretch, it must also be in very close contact with the vesicle surface. It is interersting, however, that Phe-6 is also protected from the action of receptor-linked membrane protease, which causes a rapid and specific breakdown of glucagon to turn off the signal transmission[6]. The significance of this, however, is not clear at the moment. These observations lead to the conclusion that the proteolysis expeiments here generally support the NMR results by Braun *et al.*[19] Because the micelles used for the NMR studies and the vesicle used in our proteolysis experiments have the same lipid head groups, the sturcture of glucagon located on the surface of these systems may be expected to be the same. The only difference is the radius of curvature which apparently is not a very important factor.

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New Aminothiazolyl Cephalosporins. Synthesis and Biological Evaluation of 7-[Alkoxyiminomethyl(2-aminothiazol-4-yl)acetamido]ceph-3-em-4-carboxylic Acids

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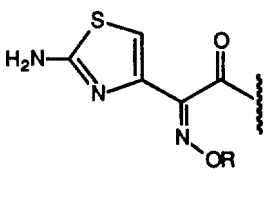
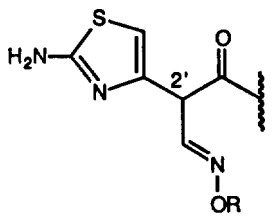
New aminothiazolyl cephalosporins with alkoxyiminomethyl(2-aminothiazol-4-yl)acetyl substituents at 7-position of cephems were synthesized starting from (2-aminothiazol-4-yl)acetate *via* one carbon homologation followed by acylation with 7-aminoceph-3-em-4-carboxylic acid derivatives. These new aminothiazolyl cephalosporins exhibit promising *in vitro* activities against various strains including Gram positive bacteria.

Introduction

Cephalosporin antibiotics with aminothiazole as a part of 7-position substituent have been increasingly popular in recent cephalosporin research.[1] In connection with our studies in developing new oral cephalosporins, we have become interested in structural modification of aminothiazole acetic acid oxime side chain at 7-position of cephalosporins. Typical structure of this 7-substituent is shown in the following formula (**I**):



(**I**)



(**II**)

It has occurred that alteration of the length of the tether chain at 2′ position might lead to change in biological activities. The simplest substituent at 7-position in cephalosporins along this line is shown in the formula (**II**). We wish to report here the synthesis of the new cephalosporins having modified substituents at 7-position of cephems which can be represented by the general formula (**II**) and their antibacterial activities.

Resutls and Discussion

The synthetic route to the aminothiazolylcephalosporins with one-carbon homologation at 2′-position is shown in Scheme 1. Starting from ethyl (2-aminothiazol-4-yl) acetate (**1**), *N*-protected compound **2** was prepared.[2] One carbon homologation at 2′-position was achieved by the reaction of **2** with methyl formate in the presence of a base followed by hydrolysis. Successful acylations by DCC promoted coupling were observed between the active esters(**5**) derived from **3** and the corresponding properly protected 7-aminocephem-4-carboxylic acid **6** to form the coupled products **7**. After acid hydrolysis of **7**, the enols **8** were reacted