Large Glucagon Immunoreactivity in Extracts of Pancreas*

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DEMETRA RIGOPOULOU, ISABEL VALVERDE,† JOSE MARCO,§ GERALD FALONA, AND ROGER H. UNGER

From the Departments of Internal Medicine and Biochemistry, The University of Texas Southwestern Medical School at Dallas, and Veterans Administration Hospital, Dallas, Texas 75216

SUMMARY

The glucagon immunoreactivity present in canine pancreatic extracts can be separated by gel filtration into two fractions; one fraction, comprising over 90% of the immunoreactivity, is similar in molecular size to the glucagon-\( ^{131}I \) marker, while the other appears to be at least twice as large. Incubation of this large glucagon immunoreactivity (LGI) in solutions such as 1 M acetic acid, 8 M urea, 0.1 M mercaptoethanol, or 6 M guanidine hydrochloride, which favor the dissociation of noncovalent protein complexes, fails to change its molecular size as determined by gel filtration, suggesting that LGI is not glucagon noncovalently bound to itself or to another protein. However, tryptic hydrolysis of LGI results in the disappearance of most of the immunoreactivity and in a reduction in the molecular size of the remaining immunoreactivity to approximately that of glucagon-\( ^{131}I \). Neither LGI nor its tryptic product, both of which resemble glucagon immunologically, has glycogenolytic activity in the perfused rat liver. It is possible that LGI includes the immunoreactive sequence of glucagon or of an immunologically similar peptide.

The recent demonstration by Steiner and colleagues that insulin is derived from a larger precursor molecule, proinsulin (1), has aroused widespread interest in the possible existence of other "prohormones." It has recently been observed that extracts of canine pancreas contain, in addition to true glucagon, a fraction of glucagon immunoreactivity which appears to be at least twice as large as glucagon (2). The following study was designed to characterize this fraction, to be referred to as large glucagon immunoreactivity, and to examine its possible relationship to glucagon.

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† Bristol Myers Research Fellow.

‡ Research Fellow of the Juan March Foundation, Madrid, Spain.

METHODS AND MATERIALS

Pancreatic Extraction Procedure—Pancreatic tissue was resected from mongrel dogs immediately after their death from Nembutal overdose. The pancreases were frozen promptly in Dry Ice and were then stored at \(-20^\circ\). Within 1 month they were extracted by the acid-alcohol method of Kenny (3). Extracts were lyophilized and stored in a vacuum at 4° until the time of use within 2 months. At that time they were reconstituted in 0.05 M \( \text{NH}_4\text{HCO}_3 \) solution at \( \text{pH} 8.8 \) so as to give an initial concentration ranging from 20 to 50 mg of extract, dry weight, per ml of solution. After at least 2 hours of grinding with a glass rod and overnight dialysis against 0.025 or 0.05 M \( \text{NH}_4\text{HCO}_3 \) solution at \( \text{pH} 8.8 \), the reconstituted extract was centrifuged at 2000 rpm for 20 min, and the sediment was discarded. The extract solution was subjected to chromatography immediately after. The specific activity of these crude extracts averaged 1 \( \mu \)g of glucagon immunoreactivity per mg of protein.

Gel Filtration Procedures—The initial chromatography of the reconstituted extract was carried out on Bio-Gel P-10 (100 to 200 mesh) columns in siliconized glass cylinders, 5 \( \times \) 115 cm. They were equilibrated in 0.05 M \( \text{NH}_4\text{HCO}_3 \) and adjusted with \( \text{NaOH} \) to \( \text{pH} 8.8 \). Following calibration of each column with blue dextran, insulin-\( ^{131}I \), and glucagon-\( ^{131}I \), 30 ml of the extract solution, containing 120 to 180 mg of protein (Lowry) and a trace of insulin-\( ^{131}I \) and glucagon-\( ^{131}I \), were subjected to column chromatography at 4°. A flow rate of approximately 1.2 ml per min was maintained and fractions of 20 ml were collected in an automatic fraction collector and stored at \(-20^\circ\).

Rechromatography of eluates was carried out as follows: each pool of eluates containing LGI or its tryptic derivatives was concentrated by lyophilization and then dialyzed overnight at 4° against 0.001 M \( \text{NH}_4\text{HCO}_3 \) and adjusted to \( \text{pH} 8.8 \) with \( \text{NaOH} \), and lyophilized. Bio-Gel P-10 (100 to 200 mesh) columns in siliconized glass cylinders, 0.9 \( \times \) 115 cm, were equilibrated with one of the following solutions: 0.05 M \( \text{NH}_4\text{HCO}_3 \) at \( \text{pH} 8.8 \), 1 M acetic acid at \( \text{pH} 2.2 \), 8 M urea at \( \text{pH} 8.1 \), 0.1 M mercaptoethanol in 8 M urea at \( \text{pH} 7.0 \), or 6 M guanidine hydrochloride at \( \text{pH} 3.7 \). A flow rate of from 0.12 to 0.21 ml per min was maintained. Columns were calibrated with blue dextran, glucagon-\( ^{131}I \), and insulin-\( ^{131}I \). The lyophilized pools of eluates were reconstituted and incubated at 25° in one of the above solutions at a concentration of 17 mg of protein per ml. After the addition of insulin-\( ^{131}I \) and glucagon-\( ^{131}I \) in tracer amounts as molecular weight markers, the pools were subjected to rechromatography at room temperature. In order
to obtain optimal resolution no more than 10 mg of protein in a
volume of 0.6 ml were applied to a column. Fractions of 1 ml
were collected in an automatic fraction collector.

**Polyacrylamide Gel Electrophoresis**—Disc electrophoresis was
carried out by the method of Davis (4) on 10% polyacrylamide
gel at room temperature in Tris-glycine buffer at pH 8.3, with
the use of a Canalco model 12 apparatus. From 20 to 40 µl of sample
containing 100 µg of protein were applied. A constant current of
4 mA per tube was maintained with a Spinco Duostat power
supply. Gels were either stained with 1% Amido black or di-
vided into 5-mm sections for glucagon assay. Each section was
placed in a tube containing 0.5 ml of assay diluent (0.2 M glycine
at pH 8.8 with 0.25% sibumun and 1:100 normal rabbit serum).
After thorough grinding of the gel with a glass rod for at least 2
hours, the mixture was centrifuged at 2000 rpm for 30 min, and
the supernatant fluid was assayed for glucagon immunoreactiv-
ity. About 95% of the applied glucagon immunoreactivity
could be recovered.

**Tryptic Hydrolysis**—Tryptic hydrolysis was carried out as
follows: a lyophilized pool of eluates containing LGI1 dissolved in
0.5 ml of 0.05 M NH4HCO3 at pH 8.8 and trypsin at a concentra-
tion of 1 mg per ml of water was added so as to give an enzyme to
protein ratio of 1:100. After gentle stirring at room temperature
for 15 min, an aliquot of the mixture was removed and stored at
-20°C until the time of glucagon assay, and the remainder was
subjected to immediate rechromatography.

**Determination of Biological Activity**—Glycogenolytic activity of
various samples in vitro was examined by measuring glucose pro-
duction by the perfused rat liver. The perfusion system and
method was modified only slightly from that used by Mortimore
(5). The perfusate consisted of a 20% suspension of outdated
human red cells, washed twice in 0.9% NaCl and twice in Krebs-
Ringer phosphate buffer at pH 7.4 and then suspended in Krebs-
Ringer phosphate buffer at pH 7.4 with 3% bovine albumin
(Cohn Fraction V). A perfusion apparatus with a Harvard perifi-
astical pump was used. Fed Sprague-Dawley rats weighing be-
drew 100 to 200 g were prepared for liver perfusion. After a
30 min equilibration period the sample to be tested, dissolved in 1
ml of assay diluent, was introduced into the drum reservoir for 60
to 80 min of perfusion. The perfusion was conducted at a con-
stant rate of 7 ml per min. One-milliliter aliquots of perfusate
were withdrawn for glucose analysis from the tubing on the portal
side of the liver at 5- and 10-min intervals throughout the per-
fusion.

Hyperglycemic activity in vivo was determined by measuring
venous glucose concentration after the portal injection of the
sample in an anesthetized dog, as previously described (6).

**Analytical Methods**—Eluates were assayed for protein by
means of absorbance at 280 nm in a DU spectrophotometer or by
the method of Lowry et al. (7), or both. Glucagon immunoreactiv-
ity of eluates was determined by the previously described radi-
immunoassay (8), in which antisera highly specific for can-
ine plasma was determined by the method of Hoffman (9) with
the use of the Technicon autoanalyzer.

**RESULTS**

**Chromatography of Canine Pancreatic Extracts**—The elution
pattern of extractable glucagon immunoreactivity of the canine
pancreas from Bio-Gel P 10 columns is shown in Fig. 1A, which
is representative of eight such experiments. More than 90% of
the immunoreactivity appeared in the zone of the glucagon-121I
marker, indicating a molecular weight in the 3500 range. A
small fraction of immunoreactivity, less than 10% of the total,
appeared before the insulin-121I marker, indicating a molecular
size at least twice that of glucagon.

To determine whether spontaneous dissociation of LGI occurs
under these conditions, a concentrated pool of eluates containing
6 mg of protein and 150 to 300 mcg eq of LGI in 0.6 ml was re-
chromatographed on a Bio-Gel P-10 column in NH4HCO3 solu-
tion at pH 8.8, as described under "Methods and Materials." Almost all the immunoreactivity remained in its original zone,
eluting before the insulin-121I marker. Fig. 1B illustrates the
elution pattern of rechromatographed LGI obtained in each of
two such experiments.

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1 The abbreviation used is: LGI, large glucagon immunoreac-
tivity.

2 Constructed by Mr. B. Moore, Vanderbilt University School
of Medicine, Nashville, Tennessee.
To determine whether self-aggregation of glucagon under these conditions might account for the small fraction of large glucagon immunoreactivity, crystalline beef-pork glucagon was subjected to rechromatography in the same manner. Rechromatography of 4 mg of glucagon in 0.5 ml of NH₄HCO₃ solution disclosed no evidence of aggregation, all of the immunoreactivity remaining in the glucagon-¹³¹I zone.

Elution Pattern of Pancreatic Glucagon Immunoreactivity in Other Species—To determine whether pancreatic extracts of other species also contain a large sized fraction of glucagon immunoreactivity, whole pancreas of duck and beef, obtained immediately after death, a human pancreas obtained at autopsy, and rat islets of Langerhans, isolated by the technique of Laccy and Kostianovsky (10), were extracted. The elution pattern of immunoreactivity of each species is shown in Fig. 2. In each case a small but significant quantity of immunoreactivity appeared in or before the zone of the insulin-¹³¹I marker. Although these represent only single experiments for each species, they suggest that large sized immunoreactivity is not confined to the dog.

Separation of Large Glucagon Immunoreactivity from Unidentified Proteins—As shown in Fig. 1A, the protein peak of the pancreatic extract is located very near the region of large glucagon immunoreactivity, raising the possibility that the high protein concentration, rather than true immunoreactivity, might have given rise to nonspecific readings in the radioimmunoassay. In order to eliminate this possibility, separation of the immunoreactivity from most of the protein was attempted by disc electrophoresis on polyacrylamide gel. The results, illustrated in Fig. 3, indicate that the immunoreactivity was not present in the sections of gel containing the most protein, Sections 1 and 2, but was present in the sections containing little or no protein, Sections 3 and 4. Large glucagon immunoreactivity does not, therefore, appear to be a nonspecific consequence of the high protein concentration of eluates in which it appears.

Biological Activity of Large Glucagon Immunoreactivity—In order to determine if LGI has biological activity, its glycogenolytic effect was determined in the perfused rat liver system of Mortimore (5). In every one of 19 experiments, perfusion of approximately 20 mpg of either crystalline beef-pork glucagon or dog glucagon was associated with an unequivocal glycogenolytic response as compared to the buffer control. Yet in each of four experiments the perfusion of 40 μg eq of LGI failed to elicit a significant glycogenolytic response; in three additional experiments 100 μg eq of LGI were also found to be inactive (Fig. 4). (This lack of response cannot be attributed to contaminating insulin, since the perfusion of insulin alone did not reduce glucose production in this liver system; furthermore, the 100 μg doses of LGI had been first treated with mercaptoethanol to destroy the insulin.)

To assess its biological activity in vivo 1 μg eq of LGI was injected endoportally in an anesthetized dog. As shown in Fig 5, LGI caused no rise in glucose, in contrast to 1 μg of crystalline beef-pork glucagon which caused a rise of 20 mg per 100. However, this LGI-containing eluate pool also contained 10 units of insulin, thus accounting for its hypoglycemic effect; it seemed possible, therefore, that a hyperglycemic effect of LGI might have been nullified by the insulin. To evaluate this possibility,
1 μg of crystalline glucagon containing 10 units of insulin was also administered. The results in Fig. 5 indicate that this amount of insulin does not prevent the initial hyperglycemic activity of crystalline glucagon.

These findings suggest that large glucagon immunoreactivity is devoid of glycogenolytic activity in vivo and in vitro.

Effect upon Large Glucagon Immunoreactivity of Solutions Favoring Dissociation of Noncovalent Protein Complexes—It seemed possible that, under the conditions prevailing during column chromatography, the LGI molecule might consist of a molecule of glucagon (mol. wt. 3485) bound noncovalently either to itself or to another protein in such a way as to render it biologically inactive. To test this possibility LGI was incubated with and rechromatographed in several solutions which favor the dissociation of protein complexes.

If LGI consisted of glucagon in complex with a basic protein, the complex should dissociate at low pH. Therefore, eluates containing 176 mg of LGI were incubated at 25° for 48 hours in 8 M urea at pH 8.1 (one experiment) and for 24 hours in 6 M guanidine at pH 3.7 (one experiment) and were then rechromatographed in the respective solution. Neither urea nor guanidine hydrochloride in high concentration are known to cause dissociation of polypeptide chains held together by noncovalent forces. Thus eluates containing 300 mg of LGI were incubated at 25° for 48 hours in 8 M urea at pH 8.1 (one experiment) and for 24 hours in 6 M guanidine at pH 3.7 (one experiment) and were then rechromatographed in the respective solution. Neither urea nor guanidine incubation caused a shift in the elution volume of immunoreactivity from the zone preceding the insulin-1311 marker in either of two such experiments.

Both urea and guanidine hydrochloride in high concentration are known to cause dissociation of polypeptide chains held together by noncovalent forces. Thus eluates containing 300 mg of LGI were incubated at 25° for 48 hours in 8 M urea at pH 8.1 (one experiment) and for 24 hours in 6 M guanidine at pH 3.7 (one experiment) and were then rechromatographed in the respective solution. Neither urea nor guanidine incubation caused a shift in the elution volume of immunoreactivity from the zone preceding the insulin-1311 marker.

The failure of acetic acid, urea, or guanidine hydrochloride to alter the molecular size of LGI makes unlikely the possibility that it represents a glucagon molecule bound noncovalently either to itself or to another protein.

Effect of Mercaptoethanol on Large Glucagon Immunoreactivity—Although glucagon itself contains no cysteine, it seemed possible that LGI might contain cysteine, perhaps as part of an interchain disulfide bridge. To exclude this possibility, a lyophilized pool of eluates containing 800 mg of LGI was incubated overnight in 0.5 ml of 0.1 M mercaptoethanol in 8 M urea and then was rechromatographed in the same solution. Again, there was no change in the elution volume of the immunoreactivity, although all of the radioactivity of the insulin-1311 marker had shifted to the zone of glucagon-1311, indicating reductive cleavage of its chains. These results indicate that LGI does not contain a cysteine residue which participates in an interchain bridge.

Effect of Trypsin on Large Glucagon Immunoreactivity—The lysine and arginine residues of glucagon render it sensitive to the action of trypsin. To determine if LGI also is sensitive, a lyophilized pool of LGI-containing eluates, containing 17.5 mg of protein and 1.5 μg eq of LGI, was dissolved in 0.5 ml of 0.05 M NH4HCO3 at pH 8.8 and was incubated with trypsin at an enzyme to protein ratio of 1:100 for 10 min at room temperature. Three such experiments were carried out. In two additional experiments at the same enzyme to protein ratio a pool of eluates containing 400 mg eq of LGI and 11.5 mg of protein was used. Reassay of the five pools after trypsin incubation revealed that 60 to 80% of the original immunoreactivity had disappeared.

From 100 to 700 mg eq of residual glucagon immunoreactivity in the trypsin digests were subjected to rechromatography and the eluates were assayed for glucagon. In each of five experiments at 15° the elution volume of the peak of residual immunoreactivity was found to have shifted and now appeared an average of 8 ml after the glucagon-1311 marker. Both canine glucagon and crystalline glucagon normally appear an average of 3 ml after the glucagon-1311 peak. A representative elution pattern of the trypsin product of LGI is shown in Fig. 6.

It is clear that limited trypsic hydrolysis brings about a major loss of large glucagon immunoreactivity and a major reduction of its residual immunoreactivity to a molecular size smaller than glucagon-1311.

More prolonged trypsin hydrolysis for 48 hours at a protein to enzyme ratio of 1:1 resulted in disappearance of at least 95% of the immunoreactivity.

Immunological Studies of Large Glucagon Immunoreactivity and Its Tryptic Products—Immunological differences between an anti-
gen and other substances which cross-react with its antibody may be reflected by a lack of parallelism of their dilution slopes as determined by radioimmunoassay. To determine if immunological differences between crystalline beef-pork glucagon, dog glucagon, dog LGI, and the tryptic product of LGI could be detected, doubling dilutions of each were assayed with an antiserum highly specific for pancreatic glucagon. As shown in Fig. 7, all dilution slopes appear to be parallel. Similar results have been obtained in two other such experiments with an antiserum highly specific for pancreatic glucagon, and in three experiments in which a less specific antiserum was used. There is, therefore, no evidence of immunological dissimilarity between any of the above substances.

Biological Activity of Tryptic Product of Large Glucagon Immunoreactivity—Since the tryptic fragment of LGI was immunologically indistinguishable from glucagon, it seemed of importance to determine if it possessed glucagon-like biological activity in the perfused rat liver system. Four experiments were conducted with 100 mg eq of the tryptic product of LGI, a dose 5 times that which, in the case of glucagon, consistently gave a striking glycogenolytic response. In none of the four experiments was a significant glycogenolytic response obtained. It is clear, therefore, that this product of LGI is not an intact glucagon molecule.

Comparison of Tryptic Product of LGI and of Glucagon—The identity of the tryptic product of LGI is obscure. The foregoing results suggest that, if the LGI molecule does contain part or all of the amino acid sequence of glucagon, its tryptic product must consist of the immunoreactive site of glucagon without a functioning biologically active site. Such a molecule has not been shown to exist.

To determine whether an immunologically active but biologically inactive fragment of glucagon could be produced, 115 μg of crystalline beef-pork glucagon was subjected to hydrolysis with 175 μg of trypsin, and was then assayed for immunological and biological activity. In two such experiments 20% of the initial immunoreactivity remained after incubation with trypsin at this enzyme to protein ratio.3 On rechromatography this residual immunoreactivity appeared as a single peak 8 ml after the peak of the glucagon-1311 marker, the zone in which the tryptic product of LGI appeared (Fig. 8). This immunoreactive fragment was then tested for biological activity. In four perfusion experiments 100 mg eq of the immunoreactivity failed to elicit a glycogenolytic response, excluding the possibility that it was undigested glucagon.

It was concluded that a biologically inactive but immunologically active fragment can be produced by limited tryptic hydrolysis of glucagon.

Discussion

The results indicate that extracts of canine pancreas, and probably of duck, beef, rat, and human pancreas as well, contain a polypeptide which reacts like glucagon with glucagon antibodies, and which is at least twice the molecular size of glucagon. Nonspecific inhibition of the reaction between glucagon and glucagon antibody by the high protein concentration of the eluates in which the immunoreactivity appears was satisfactorily excluded as the possible cause of a false peak of immunoreactivity.

Large glucagon immunoreactivity, as this fraction has been named, was found to be devoid of glycogenolytic activity in the perfused rat liver and was shown probably not to be glucagon noncovalently bound either to itself or to another protein. Nor does it appear to contain an interchain disulfide bridge. However, in the case of glucagon, cleavage of its lysine or arginine linkages (or both) by trypsin hydrolysis resulted in loss of its immunoreactivity and a reduction in molecular size of the remaining immunoreactivity to approximately that of glucagon-1311. Its lack of biological activity proved that the tryptic product could not be glucagon.

If one were to assume that all or part of the amino acid sequence of glucagon forms a part of the LGI molecule, one would then have to assume that this biologically inactive but immunologically active tryptic product of LGI is glucagon with its functional region either unexposed or absent, and that it must, therefore, be either larger or smaller than glucagon. Although resolution in the 3500 molecular weight zone of a Bio-Gel P-10 column is not adequate to permit estimation of molecular size, the consistent recovery of the immunoreactive tryptic product of LGI after the glucagon-1311 marker suggests that it is smaller rather than larger. The existence of at least one immunologically active tryptic fragment of glucagon devoid of glycogenolytic activity was convincingly established by the experiments in which
crystalline glucagon was subjected to limited trypptic hydrolysis. Bromer (11) has reported that after 2½ hours of trypsic digestion a dodecapeptide 1-19, a pentapeptide 13-17, a dodecapeptide 17-29, and an undecapeptide 18-29 are produced from crystalline glucagon (since the lysyltyrosyl bond is the most sensitive to trypsin (11), it is possible that the heptadecapeptide 13-29 was also present in the more limited 15-min digests of this study). At least one of these fragments must be the source of the immunoreactivity in the trypsic digest of glucagon. The possibility that the source of immunoreactivity in the trypsic digest of both LGI and glucagon are the same cannot be excluded since (a) they have the same elution volume, (b) they both lack biological activity, and (c) neither LGI nor its trypsic product can be differentiated immunologically from glucagon by means of dilution slope comparisons. Obviously, however, proof of their identity is not provided by this study and the composition of LGI remains to be elucidated. The issue of a precursor-product relationship between LGI and glucagon likewise is not illuminated by this study.

The demonstration of at least two or three immunoreactive peptides (LGI, its trypsic product, and the trypsic product of glucagon) which are devoid of glycogenolytic activity has important implications for the use of the glucagon radioimmunoassay. It can no longer be assumed that all of the plasma glucagon immunoreactivity measured with antisera specific for pancreatic glucagon necessarily represents intact hormone with glycogenolytic activity. Nor is it possible now to differentiate by immunochernical techniques between intact glucagon and these biologically inactive substances. The possibility that biologically inactive immunoreactive glucagon-like substances enter the circulation must now be entertained.

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