Further Characterization of a Glucagon Precursor from Anglerfish Islet Tissue[1] (36990)

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There is a growing body of evidence to indicate that glucagon, as well as insulin (1), parathyroid hormone (2), and other hormones, is synthesized via a protein precursor (3–6). In a previous report we demonstrated that in anglerfish islet tissue incubated *in vitro* with ^{3}H-tryptophan, a large molecule is initially synthesized on cytoplasmic ribosomes and is subsequently converted to glucagon within the cell. Isolation and characterization of this molecule (proglucagon) would further substantiate its role in the biosynthetic process. In the present report, some observations on the stability, electrophoretic behavior, and molecular size of anglerfish proglucagon are presented.

Materials and Methods. 1. Preparation of radioactively labeled anglerfish islet extracts. Primary and secondary islets were obtained from anglerfish during the summer months at the Marine Biological Laboratory, Woods Hole, MA. The procedures for *in vitro* incubation with radioactive isotopes and extraction of labeled proteins from incubated tissue have been described previously (3).

2. Labeled islet extracts were fractionated by gel filtration on 1.2 × 48 cm columns of Sephadex G-50 or on a 1.5 × 60 cm Bio Gel P-60 (100–200 mesh) column eluted with 1 *M* acetic acid at the rate of 15 and 20 ml/hr, respectively. Blue dextran 2000, equine cytochrome *c*, rat proinsulin, bovine insulin, porcine glucagon, and amino acids were used as molecular weight markers. Aliquots of fractions or whole fractions of column eluates were assayed for radioactivity by adding 10.0 ml of Aquasol (New England Nuclear Corp., Boston, MA) and counting in a liquid scintillation spectrometer.

3. Electrophoresis. The appropriate portions of gel filtration eluates were lyophilized and resuspended in 0.1 *M* Tris–glycine buffer (pH 7.5) for gel electrophoresis. Sample gel solution (1.7 ml) was mixed with 1.0 ml of the resuspended pool and the resulting solution was divided evenly into six 8.0 cm electrophoresis tubes for photopolymerization. Following electrophoresis in 7.5% polyacrylamide (pH 8.3), the gels were sliced into pieces approximately 1.7 mm thick. Consecutive slices from each of the five gels were pooled in liquid scintillation vials containing 1.0 ml of 0.01 *N* HCl. After 2 days, 10 ml of Aquasol were added to the slices and extracts for determination of radioactivity by liquid scintillation spectrometry.

Results and Discussion. 1. Nature and stability of precursor molecule. In the unlikely possibility that the glucagon precursor was composed of nascent glucagon bound to a terminal transfer RNA prior to its release from the ribosome, the isolated precursor and front (void volume) pools of gel filtration eluates were analyzed by spectrophotometry in the 220–300 nm range. The absorbance profiles of these pools corresponded to those of protein standards, with absorbance maxima at 278 nm. As a further check, the isolated precursor pool was treated with ribonuclease (2 μg/ml, 15 min at 22°) and refiltered. No change in the elution pattern was observed. These data indicate that the glucagon precursor had no RNA moiety.

Material in the precursor region was found to be stable in 1 *M* acetic acid, which suggests that it was in monomeric form. To substantiate this, radioactive precursor pool from

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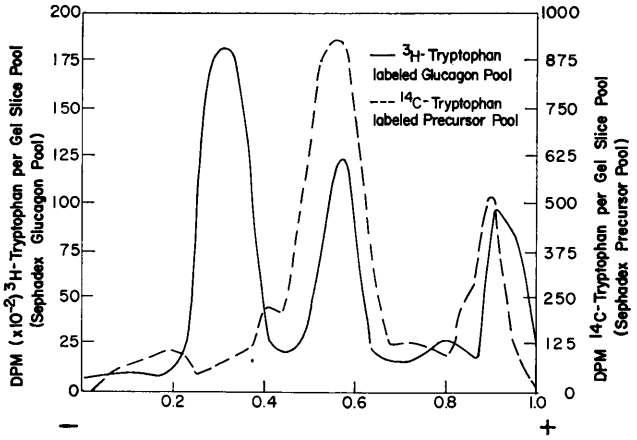

FIG. 1. Distribution of radioactivity in gel slice pools after simultaneous polyacrylamide gel electrophoresis of one Sephadex eluate pool containing anglerfish glucagon and another containing proglucagon. The glucagon pool was obtained from the filtrate of an extract of 200 mg anglerfish islet tissue incubated 4 hr with ^{3}H-(L) tryptophan (8.1 nmoles/ml, 6 Ci/mmole). The precursor pool was obtained from the filtrate of an extract of 70 mg tissue incubated 4 hr with ^{14}C-tryptophan (92 nmoles/ml, 54.5 mCi/mmole).

a gel filtration eluate, which had been stored for 6 mo at —20°, was incubated in 8 *M* urea for 96 hr at room temperature. After refiltration, control and urea-treated samples showed the same gel filtration patterns as newly isolated precursor. These data suggest that the glucagon precursor, although possessing some glucagon immunoreactivity (3), contained no glucagon in noncovalent linkages, and was eluted as a monomer on gel filtration in 1 *M* acetic acid.

2. *Electrophoretic mobility.* As noted previously (3), the glucagon precursor migrates at an R_f of approximately 0.60 under standard conditions of gel electrophoresis (7.5% polyacrylamide gel, pH 8.3), whereas glucagon and desamido glucagon migrate with an R_f of 0.32 and 0.58, respectively. In order to prove that desamido glucagon and proglucagon are separable under these conditions, a ^{3}H-tryptophan-labeled glucagon region and a ^{14}C-tryptophan-labeled precursor region from different gel filtration eluates were pooled and subjected to gel electrophoresis in the same cells. The distribution of radioactivity in the gel slices (Fig. 1) shows separation of ^{3}H-glucagon into a major band (R_f 0.31), and a minor band (R_f 0.58), and ^{14}C-proglucagon as a single band at R_f 0.56. Since the radioactive band at R_f 0.92 was present in all gel electrophoresis runs regardless of the source of the sample, we conclude that tryptophan-labeled proglucagon as isolated from Sephadex eluates is essentially homogeneous on gel

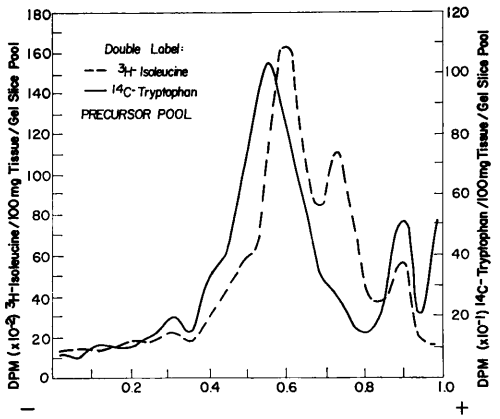

FIG. 2. Distribution of radioactivity in gel slice pools after polyacrylamide gel electrophoresis of the Sephadex eluate precursor pool from a double label incubation. The gel filtration was done on an extract of 57 mg anglerfish islet tissue incubated for 4 hr with ^{14}C-(L)-tryptophan (293 nmoles/ml, 17.1 mCi/mmole) and ^{3}H-(L)-isoleucine (1.25 nmoles/ml, 33 Ci/mmole). Tryptophan is not found in anglerfish proinsulin, and isoleucine is not a component of any known species of glucagon.

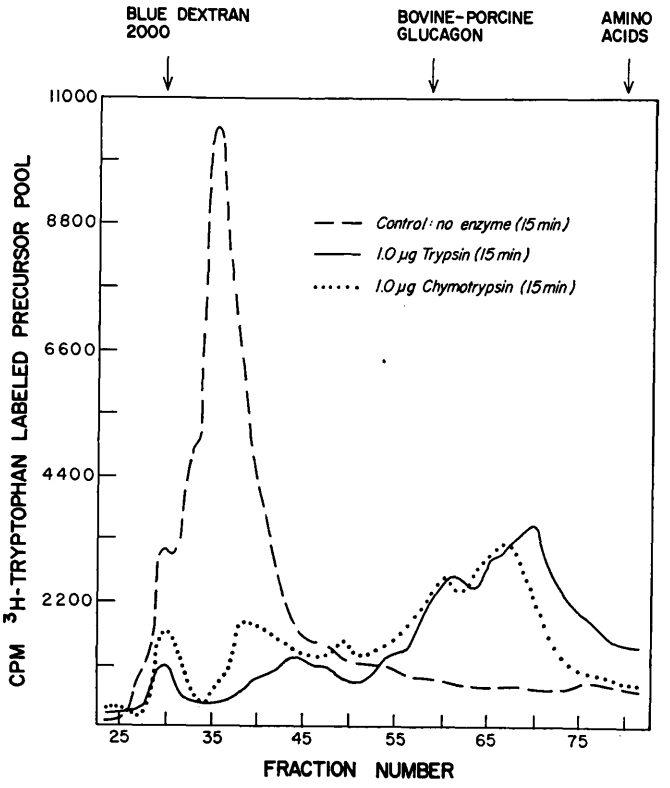

FIG. 3. The action of chymotrypsin and trypsin on anglerfish proglucagon. Precursor-containing portions of gel filtrates from ^{3}H-tryptophan-labeled extracts were pooled and lyophilized. After resuspension in 0.1 *M* Tris–glycine buffer at pH 7.8, 0.5 ml portions of the sample were treated with 1.0 μg of each enzyme. The enzyme digests were then subjected to gel filtration on Sephadex G-50 columns.

electrophoresis. On the other hand, its rate of migration was similar to that of desamido glucagon and of anglerfish proinsulin (Fig. 2) and therefore further purification is necessary before proglucagon can be used for chemical analysis.

3. Proteolytic cleavage of proglucagon. Limited trypsin hydrolysis of large immunoreactive glucagon (4, 5) and glucagon precursor (3, 6) results in cleavage to smaller peptides, one of which behaves like glucagon with respect to immunoreactivity, molecular size, and charge. The equivalence of this peptide cleavage product with glucagon has been questioned. It was therefore of interest to test the sensitivity of the large molecule to a proteolytic enzyme other than trypsin. As shown in Fig. 3, the action of chymotrypsin on the precursor was remarkably like that of trypsin. One of the peptides eluted in the same region as glucagon markers and with one of the peptide products of trypsin action. The similarity in elution profiles suggests that molecules possessing greater immunoreactivity than proglucagon, but not glucagon itself, are produced by the action of these proteolytic enzymes. Moreover, it should be pointed out that limited trypsin treatment readily destroys glucagon (3, 4). Therefore, as in the case of proinsulin–insulin cleavage (7) we suggest that the intracellular cleavage of proglucagon to glucagon probably requires a specific enzyme system within the cell.

4. Molecular weight estimation. The molecular size of anglerfish proglucagon was estimated to be greater than 9000 by gel filtration on Sephadex G 50 columns (3). A more accurate estimation was made using a

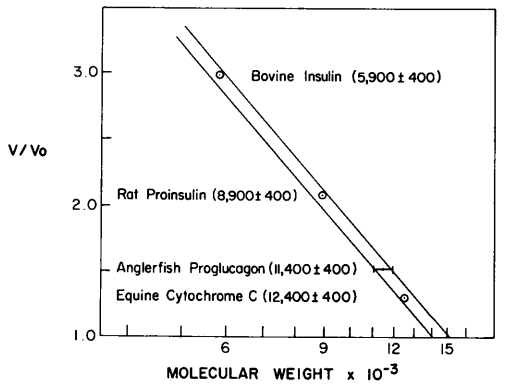

FIG. 4. Estimation of the molecular weight of anglerfish proglucagon by gel filtration. The proteins shown in the figure were used for calibration. The column was 1.5 × 60 cm of Bio-Gel P-60 (100–200 mesh) in 1 *M* acetic acid. Protein samples were dissolved in 1.0 ml of 1 *M* acetic acid. Flow rate was 20 ml/hr. V = elution volume: V_0 = void volume.

Bio Gel P-60 column with suitable molecular weight markers. As shown in Fig. 4, anglerfish proglucagon is larger than rat proinsulin and smaller than equine cytochrome *c*, and has an approximate molecular weight of 11, 400 ± 400 daltons.

The following conclusions can be drawn from these results:

1. The large molecular weight precursor in glucagon biosynthesis is a single protein which contains no ribonucleic acid.

2. The precursor molecule contains glucagon, is stable in 8 *M* urea, and withstands frozen storage for at least 6 mo. The latter property will facilitate collection of material for subsequent purification and analysis.

3. The precursor is more electronegative than glucagon, and migrates with an R_f distinguishable from anglerfish glucagon, desamido glucagon, anglerfish insulin and proinsulin.

4. The precursor is hydrolyzed by chymotrypsin and by trypsin. One of the products of both reactions has approximately the same molecular size as glucagon, but is not likely to be intact glucagon.

5. The molecular weight of the glucagon precursor in anglerfish is approximately 11, 400 ± 400 daltons.

Summary. Anglerfish proglucagon, isolated by gel filtration, was found to be stable in 8 *M* urea and to resist ribonuclease treatment, indicating its monomeric form and absence of ribonucleic acid moieties. Cleavage products of chymotrypsin-treated or trypsin-treated proglucagon are similar in molecular size. The migration pattern of anglerfish proglucagon on polyacrylamide gels (pH 8.3) indicates that the molecule is more electronegative than glucagon.

The approximate molecular weight of anglerfish proglucagon is 11,400 ± 400 daltons.

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