Proinsulin Precursors in Catfish Pancreatic Islets*

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The purpose of these experiments was to determine whether insulin-related peptides, larger than proinsulin, could be detected in pancreatic islet cells. Catfish pancreatic islets were incubated with radiolabeled amino acids. After 15- to 60-min incubation, two acid-alcohol-extractable peptides, larger than proinsulin, were detected which were approximately of $M_r$ = 12,000 and 11,000 (12 K and 11 K, respectively). They migrated as single polypeptide chains by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions, and were therefore not aggregates of insulin or proinsulin. The 12 K protein had identical mobility with catfish preproinsulin synthesized in a wheat germ cell-free system. On standard electrophoresis at pH 8.9, the 12 K protein migrated separately from proinsulin and was at least 65% one protein with two to three minor contaminants. The 12 K and 11 K proteins were chemically related to insulin and proinsulin as shown by tryptic peptide analysis, using cation exchange resin chromatography, and by two-dimensional tryptic peptide maps. Analysis of the tryptic digest of the 12 K protein, compared to proinsulin after leucine aminopeptidase treatment, suggested that the NH$_2$ terminus of the larger protein was different from that of proinsulin. These peptides were specifically bound to anti-insulin antibody. The binding was only 5 to 8% of the protein added, but was specific for the 12 K and 11 K proteins when the immunoprecipitates were examined by electrophoresis and not from contaminating proinsulin.

During the continuous incubation of the islets with [3H]leucine, 12 K and 11 K proteins were synthesized in the cell before proinsulin. When islets were first incubated with [3H]leucine for 30 min followed by incubation with excess unlabeled leucine, the 12 K and 11 K proteins appeared to show a precursor-product relationship to proinsulin and insulin. Even when total islet protein synthesis was inhibited by cycloheximide (100 μg/ml), proinsulin continued to be synthesized for up to 2 h. This suggested that the conversion of the proinsulin precursors to proinsulin in the fish is a post-translational event.

Proinsulin biosynthesis has been extensively studied in pancreatic islet tissues (1–8). Cell-free translation of mRNA from a number of islet tissues yielded a peptide larger than proinsulin, bound by anti-insulin antibody, which is called preproinsulin (9–16). Partial sequence determination of fish (14), rat (13), and bovine (16) preproinsulins revealed an extra 23 to 25 amino acids attached to the NH$_2$ terminus of proinsulin. The NH$_2$-terminal extension of these preproinsulins contains a hydrophobic region, similar to those observed with cell-free translation of mRNA for a number of peptide hormones and secretory proteins (17–27). These secretory proteins are all synthesized on polysomes bound to membranes (7, 18, 24). It has been suggested that during protein synthesis, the hydrophobic NH$_2$-terminal extension facilitates binding of the nascent chain to the membranes, and transport into the cisternae of the endoplasmic reticulum (18, 24, 25).

While extensive data on cell-free synthesis of various secretory proteins have been obtained, little is known about the synthesis and processing of these larger forms of secretory proteins by cells. Evidence for the existence of preprohormones in cells has been presented by isolating polysomes from human placental tissue (18) and from rat myeloma tumors (24). When the polysomes were stripped from membranes and incubated in vitro, the larger molecular weight precursors were synthesized. In the presence of microsomal membranes, these precursor molecules were processed to the authentic secretory proteins. The processing occurred while these peptides were still nascent chains, which explained why the precursors had not been observed in intact cells. It has been suggested, therefore, that the precursor molecules to secretory proteins would never be observed in intact cells (24). However, preproparathyroid hormone (27), pregrowth hormone (21), and precursors to proinsulin have been recently reported to be present in intact cells during short incubations (28–30).

The purpose of these experiments was to determine whether a peptide larger than proinsulin, similar to the preproinsulin described in cell-free experiments, could be observed in islet cells. The principal islet of the catfish was studied since the turnover of insulin-related proteins is 10 times slower in fish compared to mammalian islets (31). The in vitro incubation of catfish islets for shorter incubation times than previously reported (4–7, 32, 33) revealed synthesis of an acid-alcohol-extractable protein that was larger than insulin or proinsulin and identical in size with that of preproinsulin derived from cell-free translations. This protein was chemically and immunologically related to insulin and was formed in the cell before proinsulin with kinetics suggesting a precursor-product relationship.

METHODS

Incubation and Extraction of Catfish Islets—Catfish islets (Lambrich Bros. Live Fish Co., St. Louis, MO) were dissected and freed from fibrous tissue under a dissecting microscope. The islets (10 to 30 mg) were cut into eighths with a razor blade and incubated for various times in 1 ml of Krebs-Ringer buffer (pH 7.4, 2.8 mm glucose) which...
had been previously gassed with 95% O2, 5% CO2 (3). The islets were incubated at 18°C (5, 34) in the presence of labeled amino acids. The \( \text{L-}^{3} \text{H-amino acid mixture} \) \( (1.0 \text{ mCi/ml}, \text{L-}^{14} \text{C-amino acid mixture} \) \( (0.1 \text{ mCi/ml}, \text{L-[4,5-}^{14} \text{C]leucine} \) \( (1.0 \text{ mCi/ml}, \text{40 to 60 Ci/mmol}, \) and \( \text{L-[U-}^{14} \text{C]leucine} \) \( (0.1 \text{ mCi/ml}, \text{270 mCi/mmol}) \) were obtained from New England Nuclear. Each mixture consisted of purified \( \text{L-amino acids} \) in the same relative proportion as found in a typical algal protein hydrolysate. Both the \( \text{H} \) and \( \text{C} \) mixtures were of identical specific activity for double label counting. In "pulse-chase" experiments, after a period in the presence of \( \text{[H]} \)leucine, the incubation medium was removed and fresh incubation medium was added which contained unlabeled leucine \( (1.0 \text{ mM}) \) and cycloheximide at \( 100 \mu \text{g/ml} \). At the end of the incubation period, islet pieces were removed and homogenized with a Dounce homogenizer in 10% trichloroacetic acid. The insoluble material was collected by centrifuging at 1200 \( \times g \) for 15 min at 4°C and washed twice with 10% trichloroacetic acid, then once with 5% trichloroacetic acid. Th precipitate was extracted with acid-alcohol (3) for 24 h at 4°C, and then for 4 h at 37°C. The acid-alcohol-soluble material was lyophilized.

**Gel Filtration and Ion Exchange Chromatography**—The lyophilized acid-alcohol extract was dissolved in 0.5 ml of propionic acid \( (2.5 \text{ m}) \) and chromatographed on either a Bio-Gel P-60 (100 to 200 mesh, Bio-Rad Laboratories) or Bio-Gel P-100 (100 to 200 mesh) column \( (35 \times 1.6 \text{ cm}) \), eluted with 2.5 m propionic acid at a rate of 3 ml/h. Cytochrome \( c \) (1 mg) and bovine insulin (0.5 mg) standards \( (\text{Sigma}) \) were added to the column and the concentration of each was monitored by absorbance at 280 nm. 200 ml of each was collected, 25-ml aliquots of each fraction were dissolved in 5 ml of Scintiverse \( \times \) .1.6 cm), eluted with 2.5 m propionic acid \((0.9 \text{ cm}, \text{DE}52 \text{ preswollen, } \text{DE}52 \text{ preswollen, 109 to 206 mesh}) \) column \( (10 \times 0.9 \text{ cm}) \), pre-equilibrated with a buffer of 0.024 m sodium acetate, \( \text{pH} 3.3 \). The sample was applied and the column with 2 column volumes of buffer followed by a continuous sodium chloride gradient to a final sodium chloride concentration of 0.35 m, as a column of 20 ml. Proteins from the Bio-Gel P-60 column, larger than proinsulin with approximate \( M, = 11,000 \) to 12,000, were separated on a DEAE-cellulose column \( (10 \times 0.9 \text{ cm}, \text{DE}52 \text{ preswollen, microgranular, Whatman}) \) \( (10 \times 0.9 \text{ cm}) \), pre-equilibrated with a buffer of 0.024 m sodium acetate, \( \text{pH} 3.3 \). The sample was applied and the column was washed with 2,5-diphenyloxazole (PPO, scintillation grade, Packard Chemicals, 22.5 g/100 ml of dimethyl sulfoxide) for 3 h at room temperature. The 2.5-diphenyloxazole was then precipitated with distilled water and the gel was dried in a gel dryer (Hoefer Scientific Instruments). The gel was then exposed to Kodak RP-H540 X-Omat film for varying periods at \(-70^\circ \text{C}, \text{exposure time, 1 day to 3 weeks. Standard electrophoresis at pH 8.9 was performed on slab gels according to the method of Davis (39).}

**Antibody Precipitation**—Samples for assay were dialyzed against phosphate-buffered saline \( (0.15 \text{ m} \text{NaCl, 0.015 m} \text{NaH}_{2}\text{PO}_{4}, \text{pH} 7.4 \text{ for } 4^\circ \text{C}) \). Guinea pig antiguinea pig insulin antisera (diluted 1:2.5 with phosphate-buffered saline, Lot No. 526, Dr. Peter Wright, Indiana University), 100 \( \mu \text{g} \), was added to the sample in phosphate-buffered saline \( \text{pH} 7.4, 0.1 \% \text{bovine serum albumin, 0.35% Triton X-100, for 30 min at } 37^\circ \text{C. Goat anti-guinea pig } \gamma\)-globulin (Gateway Serum Co.), 100 \( \mu \text{g} \) (diluted 1:2.5), was added and the reaction allowed to proceed at \( 4^\circ \text{C} \) overnight. The precipitate was collected by centrifugation at \( 10 \times g \) for 20 min, \( 4^\circ \text{C} \), then washed three times with phosphate-buffered saline, \( 1% \text{Triton X-100, and } 2% \text{deoxycholate. The precipitate was dissolved in either NCS (Amer)-} \text{ham/} \text{Searle) and toluene, or first in 0.5 ml of 0.5 n NaOH, neutralized with 0.5 ml of 0.5 n HCl, and then in 10 ml of ACS for scintillation counting. Specific binding was defined as counts per min precipitated with anti-insulin serum minus counts per min bound to nonimmune guinea pig serum. The data were analyzed by nonpaired } t \text{ test.}

**Chromatography of Tryptic Peptides**—Mixtures of \( ^{14} \text{C-proteins with H-proteins were first reduced and amмonoethylated as described (40). The sample was dissolved in 500 } \mu \text{l of Tris/8 mera buffer, pH 8.6, gassed with N } \text{2} \text{ and reduced with 10 } \mu \text{l of } \beta\text{-mercaptoethanol for } 4 \text{ h at room temperature, then ethanolylated with ethylene} \text{m (Sigma), 30 } \mu \text{l for 30 min at room temperature. The reaction mixture}

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**Fig. 1. Bio-Gel P-100 chromatography of acid-alcohol extract of fish islet incubation. Catfish islets (10 to 30 mg) were incubated in 1 ml of Krebs-Ringer buffer, pH 7.4, 2.5 m glucose, at \( 18^\circ \text{C, } 95% O_2, 5% CO_2 \) with 1.0 \( \mu \text{Ci/ul} \) of \( [35 \text{S}]\text{leucine. The islets were removed and homogenized in 10% trichloroacetic acid, the precipitate was extracted with acid-alcohol, and the soluble fraction was lyophilized. The extract was dissolved in 500 } \mu \text{l of 2.5 m propionic acid and chromatographed on a Bio-Gel P-60 column (100 to 200 mesh) using molecular weight markers cytochrome c (} \text{M, } = 12,300\text{), bovine proinsulin (} \text{M, } = 3,000\text{), and bovine insulin (} \text{M, } = 5,700\text{). Fractions 28 to 44 were designated the } "11,000 to 12,000 M, precursor" \text{ region, Fractions 46 to 53 the } "9,000 M, proinsulin" \text{ region, and Fractions 56 to 66 the } "6,000 M, insulin" \text{ region. Approximately 450,000 cpm of labeled islet proteins from a 1-h islet incubation (C - C) and a 24-h islet incubation (C - C) were applied. V} _{\text{void}} \text{ refers to the void volume, and } V_{\text{elution}} \text{, } V_{\text{elution}} \text{, and } V_{\text{elution}} \text{ the elution volume of cytochrome c, bovine proinsulin, and insulin, respectively.}
was chromatographed on a Bio-Gel P-2 (100 to 200 mesh) column (10 × 0.9 cm) and eluted with 2.5 M propionic acid; the peptides eluting in the void volume were collected and lyophilized. Alternatively, the reduced proteins were carboxymethylated (41) with 1.1 M iodoacetamide (Sigma) at room temperature for 20 min in the dark.

The reduced and aminoethylated or the reduced and carboxymethylated proteins were digested as described above. The tryptic peptides were lyophilized, dissolved in 0.5 ml of pyridine/acetate (0.05 M, pH 7.5), applied to a Beckman AA-20 cation exchange resin column (0.9 × 20 cm) and eluted at 50°C with a continuous pyridine/acetate gradient to 2.0 M by the method of Bradshaw et al. (42). Samples (2 ml) were collected in a fraction collector. To each sample, 50 μg of bovine serum albumin carrier was added and the samples were evaporated to dryness in a vacuum oven. The residues were dissolved in 15 ml of ACS and counted on separate channels for 14C and 1H. Standard quench curves were calculated with the use of [1H]toluene and [14C]toluene standards (New England Nuclear).

Two-dimensional tryptic peptide analysis of 3H-labeled peptides was performed by electrophoresis of peptides on cellulose acetate thin layer plates (20 × 20 cm, Avicel, Analtech, Inc.). The sample was applied to the plate in 5 to 10 μl of 30% formic acid and then electrophoresed in 30% formic acid at 1000 V for 20 min. The plate was dried and then underwent ascending chromatography in H2O:acetic acid:pyridine:n-butyl alcohol (30:5:40:25, v/v). The plates were dried, impregnated with 7% 2diphenyloxazole in ether, and autodried. The chromatograms were applied to the plate in 5 to 10 μl of 30% formic acid and then electrophoresed in 30% formic acid at 1000 V for 20 min. The plate was dried and then underwent ascending chromatography in H2O:acetic acid:pyridine:n-butyl alcohol (30:5:40:25, v/v). The plates were dried, impregnated with 7% 2diphenyloxazole in ether, and autodried. The chromatograms were developed.

Cell-free Synthesis—Translation of partially purified catfish proinsulin mRNA with wheat germ extracts was performed as previously described (16). Protein determination was by the method of Lowry et al. (44).

RESULTS

Identification and Isolation of [3H]Proinsulin and [3H]Insulin

Acid-alcohol extracts of catfish pancreatic islets incubated for various times in 3H-amino-acids were first analyzed by gel filtration chromatography. Islets incubated for 1 h were compared to those incubated for 24 h. At 1 h, there was incorporation of protein migrating in the void volume and migrating with proteins the size of cytochrome c and smaller. By 24 h, incorporation appeared to have increased in the proinsulin region (Fig. 1, Fractions 46 to 53) and insulin region (Fractions 56 to 66) relative to that incorporated into total islet proteins. Since fish proinsulin synthesized by wheat germ extracts in the presence of catfish poly(A)-rich mRNA chromatographed in the M, = 11,000 to 12,000 region (Fractions 38 to 44), the labeled proteins in this region were evaluated for incorporation into protein migrating in the void volume and migrating with proteins the size of cytochrome c and smaller. By 24 h, incorporation appeared to have increased in the proinsulin region (Fig. 1, Fractions 46 to 53) and insulin region (Fractions 56 to 66) relative to that incorporated into total islet proteins. Since fish proinsulin synthesized by wheat germ extracts in the presence of catfish poly(A)-rich mRNA chromatographed in the M, = 11,000 to 12,000 region (Fractions 38 to 44), the labeled proteins in this region were evaluated for potential proinsulin precursors.

To identify the components of the precursor region, we first isolated and identified labeled catfish insulin by the methods of Yamaji et al. (5) as a standard for further comparisons.
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Catfish insulin migrates slightly ahead of marker bovine insulin by gel filtration chromatography. The $M_r = 6000$ region from the Bio-Gel P-60 column has been shown to contain glucagon, C-peptide, and immunoreactive insulins (5). The insulin region from the 24-h incubation (Fig. 1, fractions 56 to 66) was combined, lyophilized, and rechromatographed on a CM-cellulose column (Fig. 2). The nonadsorbed protein (Fig. 2A, Fractions 3 to 11) was probably C-peptide, since it was an acidic peptide which did not have a net positive charge at pH 3.3, and it also migrated with the tracker dye by standard electrophoresis, pH 8.9, (not shown) as does bovine and rat C-peptide (45, 46). Protein in Fractions 21 to 25 (Fig. 2A) migrated with porcine glucagon on standard electrophoresis, pH 8.9, as has been described for anglerfish glucagon (47) (not shown). Since marker bovine insulin eluted in Fractions 30 to 42, labeled proteins in these fractions were combined, lyophilized, and mixed with 1 mg of bovine insulin carrier. After sulfitolysis (5), the protein was rechromatographed on a Bio-Gel P-6 column (Fig. 2B). The previous single polypeptide now yielded two $^3$H-labeled peptides which migrated similarly to the sulfhydryl derivatives of B and A chains of the carrier bovine insulin, with perhaps some minor contamination of the B chain. The ratio of radioactive amino acids incorporated into the B chain and A chain was approximately 3:1, as anticipated, given the known sequence of other fish insulins (48).

The $^3$H-protein in the proinsulin region (Fig. 1, Fractions 46 to 53) was rechromatographed on Bio-Gel P-60 (Fig. 3A). As anticipated, oxidation and rechromatography of this protein did not alter its chromatographic behavior (not shown). Further evidence that this protein was a single polypeptide chain and not a partially cleaved proinsulin intermediate was the observation that it was not cleaved into two chains under reducing and denaturing conditions using SDS-gel electrophoresis (not shown). Trypsin digestion of the protein from the proinsulin region for 15 min at 37°C yielded a protein(s) which migrated ahead of bovine insulin (Fig. 3B), identical with the position of catfish insulin which, now when oxidized, was separated into two peptides (Fig. 3C). These data suggested that the major component of the proinsulin region was intact proinsulin.

**Characterization of Proteins in the Precursor Region**

**SDS-Urea Polyacrylamide Gel Electrophoresis**—Labeled proinsulin precursors, if present, should be enriched relative to proinsulin after relatively short incubation times. To examine this possibility, $^3$H-islet proteins were studied by SDS-urea electrophoresis optimized for resolution of proteins of $M_r$ = 2,000 to 20,000. The acid-alcohol-soluble proteins labeled with $[{}^3$H]leucine during a 30-min incubation were electrophoresed; the resultant autoradiograph of the dried gel is seen in Fig. 2. Two major proteins larger than proinsulin with estimated molecular weights of 12,000 and 11,000 were observed. These proteins have been designated 12 K and 11 K, respectively.

The $^3$H-labeled 12 K and 11 K proteins were isolated for further study. $^3$H-labeled 12 K protein was obtained from islets incubated with a mixture of labeled amino acids for 30 to 90 min, chromatographed on Bio-Gel P-100, and rechromatographed to a single peak. SDS-urea electrophoresis of this material indicated that the 12 K protein co-migrated with preproinsulin synthesized by a wheat germ cell-free extract in the presence of catfish islet mRNA (Fig. 5A). The cell-free extract of cattfish islet incubation. Catfish islets were incubated in 2 mCi/ml of $[{}^3$H]leucine for 30 min, homogenized in 10% trichloroacetic acid, and extracted with acid-alcohol as before (Fig. 1). The acid-alcohol extract was dissolved in SDS-gel electrophoresis sample buffer and electrophoresed on an SDS-8 M urea 17.5% polyacrylamide separating slab gel with a discontinuous buffer system as described under “Methods.” Standard proteins including ovalbumin (Ova) ($M_r = 43,000$), chymotrypsinogen (Chymo) ($M_r = 23,200$), myoglobin (Myo) ($M_r = 17,200$), cytochrome c (Cyto) ($M_r = 12,000$), porcine proinsulin (PI) ($M_r = 9,000$), and bovine insulin (I) ($M_r = 5,700$) were visualized by staining with Coomassie blue and destaining in 5% methanol, 7% acetic acid. The $[{}^3$H]leucine-labeled proteins were visualized by autoradiography as described under “Methods.” The gel was exposed to x-ray film for 1 week at -70°C.

**Fig. 4.** SDS-urea electrophoresis of $[{}^3$H]leucine-labeled acid-alcohol extract of catfish islet incubation. Catfish islets were incubated in 2 mCi/ml of $[{}^3$H]leucine for 30 min, homogenized in 10% trichloroacetic acid, and extracted with acid-alcohol as before (Fig. 1). The acid-alcohol extract was dissolved in SDS-gel electrophoresis sample buffer and electrophoresed on an SDS-8 M urea 17.5% polyacrylamide separating slab gel with a discontinuous buffer system as described under “Methods.” Standard proteins including ovalbumin (Ova) ($M_r = 43,000$), chymotrypsinogen (Chymo) ($M_r = 23,200$), myoglobin (Myo) ($M_r = 17,200$), cytochrome c (Cyto) ($M_r = 12,300$), porcine proinsulin (PI) ($M_r = 9,000$), and bovine insulin (I) ($M_r = 5,700$) were visualized by staining with Coomassie blue and destaining in 5% methanol, 7% acetic acid. The $[{}^3$H]leucine-labeled proteins were visualized by autoradiography as described under “Methods.” The gel was exposed to x-ray film for 1 week at -70°C.

**Fig. 5.** SDS-urea electrophoresis of fish islet proteins labeled with $[{}^3$H]leucine and purified as described under “Methods.” A, comparison of purified $[{}^3$H]leucine-labeled protein ($M_r = 12,000$) (30-min incubation, Lane 1) with $[{}^3$S]methionine preproinsulin synthesized by a wheat germ extract in the presence of islet mRNA (Lane 2) (see “Methods”). $B$, electrophoresis of purified $[{}^3$H]leucine-labeled protein ($M_r = 11,000$) (Lane 1), proinsulin (Lane 2), and insulin (Lane 3), all obtained from a 24-h incubation. Electrophoresis was performed as described in the legend to Fig. 4. Ova, ovalbumin; Chymo, chymotrypsinogen; Myo, myoglobin; Cyto, cytochrome c; PI, porcine proinsulin; I, bovine insulin.
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The mobilities of the labeled islet proteins with and without reduction were compared to that of preproinsulin synthesized by the wheat germ cell-free protein. Proinsulin was retarded on reduction and could not be distinguished from the 11 K protein. The proinsulin band contained predominantly intact proinsulin since reduction of the disulfide bridges did not produce any smaller peptides (not shown).

Because both gel filtration and SDS-electrophoresis separate proteins predominantly by size, the 12 K protein and proinsulin were electrophoresed on polyacrylamide gels at pH 8.9 by the method of Davis (39) as seen in Fig. 6A, the 12 K protein was heterogeneous, with one major protein comprising 65% of the total. The proinsulin peak comprised 80% of the total 9 K material electrophoresed (Fig 6B). There was essentially no proinsulin observed in the 12 K protein.

Tryptic Peptide Analysis of "H-labeled 12 K and 11 K Proteins—The chemical relationship of the higher molecular

product has been identified as preproinsulin, a peptide with approximately 23 amino acids on the NH₂ terminus of proinsulin, by tryptic peptide analysis with proinsulin (16), as well as sequence analysis of the NH₂ terminus (13–15). Less than 10% contamination with 11 K protein and no detectable proinsulin was observed in the rechromatographed material. "H-labeled 11 K protein was isolated from the precursor region of a 24-h islet incubation on Bio-Gel P-60 chromatography, rechromatographed to a single peak on Bio-Gel, and then chromatographed on a DEAE-cellulose column. The mobilities of purified protein 11 K, proinsulin, and insulin fractions on SDS-urea polyacrylamide gel are seen in Fig. 5B. The

Fig. 6. Densitometric tracings of radioautographs of "H-labeled 12 K and 9 K proteins electrophoresed on standard polyacrylamide gels at pH 8.9. A, ["H]leucine-labeled 19 K protein obtained after 30 min islet incubation and chromatographed to a single peak by Bio-Gel P-100 chromatography, was electrophoresed by the method of Davis (39). The gels were impregnated with 2,5-diphenyloxazole, dried, and a radioautograph was obtained as described under "Methods." The radioautographs were scanned with a Gilford 2400S gel scanner at 550 nm. B, densitometric scan of ["H]leucine labeled 9 K protein obtained as in Fig. 3.

Fig. 7. Comparison of the tryptic peptides of catfish proinsulin and insulin with those of the Mᵣ = 11,000 and 12,000 proteins after reduction and aminoethylation. Islets were incubated in either "C-amino-acid mixture or "H-amino-acid mixture of identical specific activity for double-label counting. A, the ["C]insulin was mixed with ["H]proinsulin, reduced, aminoethylated, digested, and eluted from a Beckman AA-20 cation exchange resin column using a continuous pyridine/acetate gradient as described under "Methods." Two-milliliter samples were collected, dried in a vacuum oven, and counted. Tryptic peptides of ["C]insulin (○ — ○); tryptic peptides of ["H]-proinsulin (○ — ○). Arrow indicates start of pyridine/acetate gradient. B, tryptic peptides of "H-labeled Mᵣ = 11,000 protein (△ — △); tryptic peptides of "H-labeled Mᵣ = 11,000 protein (○ — ○). C, tryptic peptides of ["H]-labeled Mᵣ = 12,000 protein (□ — □); tryptic peptides of ["C]proinsulin (○ — ○).
sulin peptides, a two-dimensional analysis of the tryptic peptides of these proteins was performed (Fig. 8). The autoradiographs showed seven tryptic peptides from the 9 K and 12 K proteins that suggested that proinsulin, 11 K protein, and 12 K protein were substantially similar and excluded the possibility of large proteins with identical mobility. These peptide analyses suggested that the N\textsubscript{H}\textsubscript{2} terminus of the 12 K protein and proinsulin were located on the NH\textsubscript{2} terminus of proinsulin, as has been observed for preproinsulins synthesized in cell-free systems. Leucine aminopeptidase was used to modify the NH\textsubscript{2} termini of both proteins. The conditions of the tryptic analysis were different than the one just described. The proteins were labeled with [\textsuperscript{3}H]leucine and, instead of aminoethylation, the proteins were first carboxymethylated. Digestion of carboxymethylated proinsulin should produce A chain, desoctapeptide B chain, and connecting peptide (C-peptide) as the major products. Fish C-peptide may also have extra trypsin-sensitive bonds (48). Proinsulin and 12 K protein not treated with leucine aminopeptidase again showed overlap of major peptides (Fig. 9A). When the identical proteins were first reacted with aminopeptidase for 8 h before digestion, the peptide elution pattern now showed a new peptide present in the tryptic peptides of both proinsulin and 12 K protein (Fig. 9B, Fractions 56 to 58) which was not seen in either previously. This may be free leucine. There was also a peptide seen in proinsulin (Fig. 9B, Fractions 50 to 53) which was not present in the tryptic peptides of the 12 K protein. This suggested that the NH\textsubscript{2} terminus of the 12 K protein and proinsulin were different.

**Immunchemical Studies**—Binding of catfish insulin, proinsulin, 11 K and 12 K proteins to anti-bovine insulin antiserum was determined. Specific binding of [\textsuperscript{3}H]-protein was defined as that bound by anti-insulin antiserum minus that bound by nonimmune serum. Approximately 40% of the weight proteins to proinsulin and insulin was determined by tryptic peptide analysis. The islets were incubated in \textsuperscript{3}H-amino-acid mixture and \textsuperscript{14}C-amino-acid mixture of the same specific activities, and the proteins were isolated as above. As standards, the tryptic peptides of [\textsuperscript{14}C]insulin and [\textsuperscript{3}H]proinsulin eluted from a Beckman AA-20 cation exchange resin column were compared (Fig. 7A). Since the cystines were reduced and aminoethylated, trypsin digestion of the insulin would be expected to produce four major peptides from the B chain and three from the A chain, as well as free asparagine. Catfish proinsulin should contain these tryptic peptides plus the C-peptide and three arginines. There was overlap of at least seven peptides, with extra peptides in proinsulin, as expected. Since the elution of the tryptic peptides varied somewhat for each preparation, as well as the extent of digestion, [\textsuperscript{14}C]proinsulin was added as an internal marker prior to digestion with each subsequent analysis. Tryptic peptide analysis of \textsuperscript{3}H-labeled 11 K protein using [\textsuperscript{14}C]proinsulin as an internal control (Fig. 7B) showed overlap of all the major peptides in the 11 K protein with proinsulin. A comparison of \textsuperscript{3}H-labeled 12 K protein with [\textsuperscript{14}C]proinsulin (Fig. 7C) revealed peptides in the 12 K protein similar to those in proinsulin.

To further substantiate the 12 K protein-contained proinsulin peptides, a two-dimensional analysis of the tryptic peptides of these proteins was performed (Fig. 8). The autoradiographs showed seven tryptic peptides from the 9 K and 12 K proteins with identical mobility. These peptide analyses suggested that proinsulin, 11 K protein, and 12 K protein were substantially similar and excluded the possibility of large amounts of contaminating nonproinsulin proteins.

It was next determined whether extra amino acids of the 12 K protein were located on the NH\textsubscript{2} terminus of proinsulin, as has been observed for preproinsulins synthesized in cell-free

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**Fig. 8.** Tryptic peptide analysis of [\textsuperscript{3}H]leucine-labeled proteins. [\textsuperscript{3}H]leucine-labeled proteins (obtained as in Fig. 5) were oxidized with performic acid, lyophilized, and then digested as in Fig. 7. The samples were electrophoresed in 30% formic acid at 1000 V for 20 min, the paper was dried, and then chromatography was performed as described under "Methods." Gels were impregnated with 7.5% 2,5-di-phenyloxazole in ether, and radioautographs were obtained after exposure to X-Omat film for 2 weeks. A, radioautograph of oxidized and trypsin-digested [\textsuperscript{3}H]leucine-labeled 9 K protein. B, radioautograph of oxidized and trypsin-digested [\textsuperscript{3}H]leucine-labeled 12 K protein. C and D, representation of tryptic peptides from A and B, respectively. Peptides that have the same mobility relative to DNP-lys in the 12 K and the 9 K proteins are shaded. △ is origin. DNP-lys, 2,4-dinitrophenyl lysine standard.
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Fig. 10. SDS-urea electrophoresis of immune precipitates. Catfish islets were incubated for 60 to 90 min in [3H]leucine and the acid-alcohol extract was separated by Bio-Gel P-100 column chromatography into crude $M_r = 12,000$ protein, $M_r = 11,000$ protein, and proinsulin. The radioactive proteins were dialyzed against phosphate-buffered saline, pH 7.4, at 4°C for 16 h. Identical fractions were then incubated with guinea pig antibovine insulin antiserum (100 µl at 1:2 dilution) and the antibody-antigen complex was precipitated by incubating with goat anti-guinea pig y-globulin (300 µl undiluted) for 16 h at 4°C. The precipitates were washed three times in phosphate-buffered saline, 1% SDS, 1% deoxycholate, and then extracted with 500 µl of acid-alcohol for 6 h at 37°C. The acid-alcohol-extracted material was lyophilized and electrophoresed in SDS-urea as before (Fig. 4). The gels were cut into 1.5-mm sections and digested in 0.5 ml of 0.5 N NaOH for 1 h at 90°C, and then neutralized with 0.5 ml of 0.5 N HCl and counted. Standards were stained separately from the cut gels. A, [3H]leucine and [3H]proinsulin extracted from [3H]leucine-labeled islets was precipitated. The specific binding of 12 K and 11 K proteins which contain proinsulin and insulin peptides were in fact converted by the islet cell to proinsulin and insulin. Islets were therefore either continuously labeled with [3H]leucine for up to 4 h or labeled for 30 min followed by incubation in unlabeled leucine for up to 3% h, and the proteins were separated by SDS-urea electrophoresis. In the continuous labeling experiment, there was linear incorporation of radioactivity with time. An example of incorporation during 15 min compared to 60 min is seen in Fig. 11A. At 15 min, there was relatively more incorporation into 12 K protein compared to that in proinsulin, while by 60 min, the major protein synthesized was proinsulin. Similarly in the pulse-chase experiment (Fig. 11B), at 30 min, the major fraction of proinsulin and 11 K and 12 K [3H]leucine-labeled proteins to an antiglucagon antiserum was tested and no specific binding was observed under the same conditions as were used for the anti-insulin antiserum.

Conversion of the 12 K and 11 K Proteins to Proinsulin and Insulin

We next sought to determine whether the 12 K and 11 K proteins which contain proinsulin and insulin peptides were in fact converted by the islet cell to proinsulin and insulin. Islets were therefore either continuously labeled with [3H]leucine for up to 4 h or labeled for 30 min followed by incubation in unlabeled leucine for up to 3% h, and the proteins were separated by SDS-urea electrophoresis. In the continuous labeling experiment, there was linear incorporation of radioactivity with time. An example of incorporation during 15 min compared to 60 min is seen in Fig. 11A. At 15 min, there was relatively more incorporation into 12 K protein compared to that in proinsulin, while by 60 min, the major protein synthesized was proinsulin. Similarly in the pulse-chase experiment (Fig. 11B), at 30 min, the major fraction of proinsulin and 11 K and 12 K [3H]leucine-labeled proteins to an antiglucagon antiserum was tested and no specific binding was observed under the same conditions as were used for the anti-insulin antiserum.

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newly synthesized protein was in the 12 K region, while after a 60-min chase, a decrease of [3H]labeled 12 K protein was accompanied by an increase in the radioactivity of the 11 K region and in proinsulin. Realizing that the 12 K protein was heterogeneous and that there was some overlap of these proteins (Fig. 11), an approximation of the appearance and disappearance of [3H]leucine in the 12 K region, 11 K region, proinsulin, and insulin during a continuous incubation or pulse-chase experiment was made (Fig. 12). Labeled protein in the 12 K region appeared first, and incorporation had reached a steady state by 90 min. Similarly, [3H]leucine incorporation into the 11 K region plateaued, while [3H]proinsulin continued to accumulate during the 240 min of the experiment. [3H]Insulin was first detected at 120 to 180 min. In the pulse-chase experiment islets were incubated in [3H]leucine for 30 min, followed by a chase in unlabeled leucine. [3H]labeled $M_r = 12,000$ protein (●—●); [3H]labeled $M_r = 11,000$ protein (□—□); [3H]proinsulin (○—○); [3H]insulin (△—△).

FIG. 12. Kinetics of insulin precursors. SDS-urea polyacrylamide slab gels were run as in Fig. 11 and were sliced and counted. The area under each section was summed at each time interval, and the radioactivity incorporated was expressed as counts per min/μg of total acid-alcohol-extractable protein. A, continuous labeling in islet incubation with [3H]leucine. B, pulse-chase, islets incubated with [3H]leucine for 30 min, followed by a chase in unlabeled leucine. [3H]labeled $M_r = 12,000$ protein (●—●); [3H]labeled $M_r = 11,000$ protein (□—□); [3H]proinsulin (○—○); [3H]insulin (△—△).

observed, with proinsulin continuing to appear for up to 2 h in spite of inhibition of total islet protein synthesis.

Discussion

An acid-alcohol-soluble protein slightly larger than proinsulin has been detected in intact catfish pancreatic islet cells. Evidence that it is a proinsulin precursor is as follows: 1) it was a single polypeptide chain under reduced conditions on SDS-urea polyacrylamide gels with an estimated molecular weight of 12,000, identical in mobility to preproinsulin synthesized in a cell-free system. In contrast, proinsulin had mobility corresponding to $M_r = 9000$ under identical conditions; 2) the 12 K protein contained proinsulin tryptic peptides when analyzed by ion exchange chromatography and two-dimensional peptide analysis; 3) the 12 K protein was slightly, but significantly, bound by anti-insulin serum and the immunoprecipitated protein was not due to contaminating proinsulin; 4) the 12 K protein was synthesized before proinsulin and, in pulse-chase experiments, had a half-time of disappearance of 15 to 30 min while proinsulin continued to be synthesized. The structure of this protein has yet to be determined.

An 11 K protein was also observed which contained tryptic peptides of proinsulin. It was specifically bound by anti-insulin antibody. We are not sure of the kinetics of conversion since the 11 K protein was partially obscured by 12 K protein in the polyacrylamide gel analysis even at the earliest incubation times (see Fig. 11B). The 11 K protein may be an intermediate in the processing of preproinsulin, or it may be a partially cleaved and uncleaved form of proinsulin. Thus, when proinsulin was reduced and analyzed by SDS-gel electrophoresis, the proinsulin co-migrated with the 11 K protein (not shown). The existence of a transient 11 K intermediate can be resolved only when the proteins are sequenced. The COOH termini of the 12 K and 11 K proteins have not yet been evaluated, and it is possible that there is a carboxyl extension as observed with proglucagon (49).

Tryptic peptide analysis was necessary to confirm that the 12 K protein might be a proinsulin precursor synthesized in an intact cell. First, the identity of labeled insulin and proinsulin had to be obtained as standards for comparison. Labeled islet proteins were separated by gel filtration and ion exchange chromatography. Insulin was identified as a $M_r = 6000$ polypeptide bound by anti-insulin antibodies which, after sulfitolysis, separated into two chains which co-migrated with B and A chains of bovine insulin. Proinsulin was identified as a single peptide of $M_r = 9000$ under reducing conditions, bound by anti-insulin serum. This material when oxidized had unchanged mobility. After mild trypsin treatment, it co-migrated with catfish insulin on gel filtration chromatography. The digested material, when subsequently oxidized, separated into two peaks which co-migrated with the B and A chains of bovine insulin. In this way, it was demonstrated by gel filtration chromatography that the majority of the material in the presumed proinsulin region was intact proinsulin and not proinsulin intermediates. At least seven tryptic peptides of [15]C(proinsulin and [15]Hinsulin co-eluted from the ion exchange resin (Fig. 7A). The 11 K tryptic peptide profile looked very similar to that of proinsulin, with only minor differences noted. If the 11 K protein is a partially processed intermediate between the 12 K protein and proinsulin, only a small difference between the 11 K protein and proinsulin would be anticipated. Comparison of the tryptic peptides of the 12 K protein with labeled proinsulin (Fig. 7C) showed that the 12 K protein contained tryptic peptides of proinsulin. The sequence of the precursor region for fish propreinsulin has not been elucidated. Comparison to known sequences of other...
precursor proteins would suggest that there may be one or two trypsin-sensitive bonds in this region. The presence of the hydrophobic residues on the NH2 terminus of the 12 K protein may alter the ability of the protein to be digested. This was suggested by the two-dimensional tryptic peptide analysis of proinsulin and the 12 K protein seen in Fig. 8. For instance, a trypptic peptide was observed in proinsulin which moved toward the anode under the very acidic conditions of electrophoresis (Fig. 8, A and C). This probably represented sulfonated A chain. In contrast, this peptide was not observed in the 12 K protein (Fig. 8B). Tryptic peptide analysis of bovine preproinsulin showed an absence of A chain (15). Furthermore, tryptic peptides were present in proinsulin which were not observed in the 12 K protein. Again, this may be explained by the fact that the 12 K protein may have different folding characteristics and different susceptibility to trypsin digestion. Alternatively, some of the trypptic peptides of the 12 K protein may be insoluble and lost during preparation. Some differences of the trypptic peptide maps may be expected since the 12 K protein was known to be contaminated to some extent by other polypeptides (see below).

Since a 12 K precursor to glucagon has been described in anglerfish islets (7), our 12 K protein was further subjected to analysis by electrophoresis on polyacrylamide gels at pH 8.9. Separation by charge revealed that while one protein made up the majority of the material, there were at least two or three other minor components. The previously identified proglucagon was detected by labeling with tryptophan, an amino acid present in glucagon-related peptides, but not in insulin or proinsulin. Other amino acids preferentially labeled proinsulin suggesting that in fish islets, proglucagon synthesis is considerably less extensive relative to proinsulin. Furthermore, we were unable to detect any leucine-labeled or amino acid mixture-labeled 12 K protein binding to antiglucagon antibody; this, however, does not rule out the possibility that one of the minor components of the 12 K protein may be proglucagon. A prosomatostatin has tentatively been observed in fish islets as well (50).

Binding of the proinsulin precursors to anti-insulin antibody was small, but significantly greater than to nonimmune serum, and the specificity was confirmed by SDS-gel electrophoresis of the immune precipitates. The low affinity was anticipated since the binding of preproinsulin, synthesized in cell-free systems, has been reported to be only 20 to 30% of the total radioactivity in the preproinsulin region for rat preproinsulin (13). Only 10% of anglerfish preproinsulin was bound to anti-insulin antibody (14). Bovine preproinsulin was bound specifically by antiovine insulin antibody only to 0.52% (15). None of these preparations have been checked by standard electrophoresic to determine heterogeneity. Catfish proinsulin precursors had similar low binding, 5 to 8% with the highest concentrations of antibody used. Antibovine insulin antibody was used, and the affinity of the antibody for fish insulins is considerably less than that of mammalian insulin. From the results of Lomedico et al. (15), it appeared that binding of preproinsulin by anti-insulin antibody was facilitated by antipremoisulin antibodies formed when crude insulin was used as an antigen.

The conversion of the 12 K protein to proinsulin was studied by SDS-gel electrophoresis, although the 12 K band was heterogeneous by standard electrophoresis. Since contaminating proteins comprised, at most 35% of the labeled protein in the 12 K band, a reduction of label in this region, following 30-min chase to one-third the initial level, represents an approximation of the turnover of the major component, the proinsulin precursor. The turnover of the minor components has not been evaluated.

The conversion of the 12 K protein to proinsulin, even in the presence of cycloheximide, appeared to be a post-translational event as opposed to a slow translation of the fish proinsulin mRNA at 18°C. While it appears clear that pre-IgG light chains and pre-hPL are processed as nascent chains (18, 24), this may not be the case for all secretory proteins. If, in fact, presecretory proteins are cleaved as nascent chains, then the preforms would never be observed in vivo. This is so for most secretory proteins investigated in vivo; yet, prepro-PTH (27) and preproinsulin (28-30) have been detected in cells, suggesting that perhaps these hormones are processed after translation. It is interesting to note that the size of pre-IgG light chain and pre-hPL (approximate M, = 25,000) is over twice the size of prepro-PTH and preproinsulin and, therefore, it is possible that the smaller proteins can be released from the polysomes before processing, whereas the larger proteins are processed while still on the ribosomes.

The steps in the conversion process have yet to be determined. Anglerfish preproinsulin was converted to proinsulin in the presence of microsomal membranes (14) and no intermediate was seen, but the incubation was for 90 min and no shorter times were reported. Further work in analyzing the sequence of the NH2 terminus of the 12 K precursor and the apparent intermediate would further clarify the steps in the conversion process.

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