

Characterization of Proinsulin- and Proglucagon-converting Activities in Isolated Islet Secretory Granules

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ABSTRACT The conversion of proglucagon and proinsulin by secretory granules isolated from both prelabeled and unlabeled anglerfish islets was investigated. Either granules isolated from tissue labeled with [³H]tryptophan and [¹⁴C]isoleucine or [³⁵S]cysteine, or lysed granules from unlabeled tissue to which exogenously labeled prohormones had been added were incubated under various conditions. Acetic acid extracts of these granule preparations were analyzed for prohormone and hormone content by gel filtration. Both prelabeled and lysed, unlabeled secretory granules converted radiolabeled precursor peptides (*M_r* 8,000–15,000) to labeled insulin and glucagon. The accuracy of the cleavage process was established by demonstrating comigration of products obtained from *in vitro* cleavage with insulin and glucagon extracted from intact islets using electrophoresis and high-pressure liquid chromatography (HPLC). The pH optimum for granule-mediated conversion was found to be in the range of pH 4.5–5.5. Conversion of both proglucagon and proinsulin by secretory granules was significantly inhibited in the presence of antipain, leupeptin, *p*-chloromercuribenzoate (PCMB) or dithiodipyridine (DDP) but not chloroquine, diisopropyl fluorophosphate, EDTA, *p*-nitrophenyl guanidino-benzoate, soybean trypsin inhibitor, or *N*-*p*-tosyl-L-lysine chloromethyl ketone HCl. The inhibitory action of PCMB and DDP was reversed in the presence of dithiothreitol. Both membranous and soluble components of the secretory granules possessed significant converting activity. HPLC and electrophoretic analysis of cleavage products demonstrated that the converting activities of the membranous and soluble components were indistinguishable. The amount of inhibition of proinsulin and proglucagon conversion caused by 600 μg/ml porcine proinsulin was significantly lower than that caused by the same concentration of unlabeled anglerfish precursor peptides. These results indicate that the proinsulin and proglucagon converting enzyme(s) in the anglerfish pancreatic islet is a unique intracellular thiol proteinase(s) that may be granule membrane-associated and may require the presence of prohormone sequences in addition to the dibasic residues at cleavage sites for substrate recognition and/or binding.

Although the subcellular conversion of proinsulin to insulin has been the subject of numerous investigations (1–9), the mechanisms of proglucagon biosynthesis and conversion at the subcellular level have been the focus of only one study. Using

anglerfish pancreatic islet, Noe et al. (7) demonstrated that both proglucagon and proinsulin are synthesized in the microsomes and then transported to the secretory granules. The appearance of radiolabeled glucagon and insulin in the micro-

some fraction suggested that the processing of both prohormones to their respective products begins in the endoplasmic reticulum/Golgi complex. Conversion of both prohormones was detected in the secretory granules as well, indicating that the enzyme(s) capable of converting the precursor peptides was also transported to secretory granules.

In the experiments described herein, the nature of converting enzyme activity(-ies) was examined in isolated secretory granules by monitoring the ability of lysed granule preparations to convert labeled prohormones in the presence or absence of specific proteinase inhibitors. The possibility that the converting enzyme(s) may be associated with secretory granule membranes was examined by comparing converting activity of the membranous and soluble components of isolated secretory granules. Finally, the selectivity and specificity of the converting enzyme(s) were assessed by testing the ability of an artificial substrate, porcine proinsulin, to inhibit the conversion process. Analysis of granule-mediated conversion of prosomatostatin to somatostatin has been presented in a separate communication (10). Portions of this work have appeared in the form of an abstract (11).

MATERIALS AND METHODS

Materials

L-[³H]tryptophan (5.96 Ci/mmol), L-[³⁵S]cystine (483.59 Ci/mmol), and L-[¹⁴C]isoleucine (296 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.). ¹²⁵I-glucagon was obtained from Nuclear Medical Laboratories, Dallas, Tex. Bio-Gel P-10 (100–200 mesh) and Protein Reagent Kit were purchased from Bio-Rad Laboratories (Rockville Center, N. Y.). Preblend 3a70B liquid scintillation fluid was obtained from Research Products International (Elk Grove Village, Ill.). All reagents and embedding medium for electron microscopy were purchased from Electron Microscopy Sciences (Fort Washington, Pa.). Proteinase inhibitors, *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide, and 5'-Nucleotidase Reagent Kit were obtained from Sigma Chemical Co. (St. Louis, Mo.). Antipain and leupeptin were gifts from Dr. Walter Troll, New York University Medical Center and the Japanese-American Cancer Program, New York. Porcine proinsulin was generously supplied by Dr. Ronald Chance, Lilly Research Laboratories, Indianapolis, Ind.

Incubation of Islet Tissue

Pancreatic islets were removed from anglerfish (*Lophius americanus*) obtained at the Marine Biological Laboratory, Woods Hole, Mass. After decapsulation, islet tissue was either sliced into 2-mm³ pieces for incubation or homogenized in 1.0 ml of 0.25 M sucrose containing 1 mM MgCl₂ for fractionation. Tissue slices were subjected to a 30-min preincubation at 20°C in 3.0 ml of Krebs-Ringer bicarbonate buffer (KRB), pH 7.4, under 95% O₂/5% CO₂. After removal of the preincubation medium, the tissue was incubated in 1–2 ml of KRB containing 100 μ Ci of [³H]tryptophan and either 10 μ Ci of [¹⁴C]isoleucine or 50 μ Ci of reduced [³⁵S]cystine. After various periods of incubation, islet tissue was washed twice with fresh KRB and then homogenized in 0.25 M sucrose containing 1 mM MgCl₂.

Subcellular Fractionation

Fractionation of the islet tissue was performed as previously described by Noe et al. (12), except that all sucrose solutions contained 1 mM MgCl₂ to maintain ribosomal integrity and association with microsomal membranes. This procedure resulted in seven subcellular fractions: F-I (nuclei); F-II_r (mitochondria); F-II_m (mitochondria, cell debris); F-II_B (secretory granules 220–310 nm in diameter); F-III (microsomes); F-III_C (secretory granules 150–200 nm in diameter); and F-IV (postmicrosomal supernate). For most of the experiments performed in the present study, the two secretory granule fractions (F-II_B + F-III_C) were combined and sedimented by centrifugation for use in conversion assays.

Incubation of Isolated Secretory Granules

Secretory granules were suspended in 0.02 M sodium acetate-acetic acid buffer, pH 5.2, and then gently mixed by hand in a Potter-Elvehjem homogenizer (Kontes Co., Vineland, N. J.). For some experiments, granules were suspended

in acetate-buffered 0.25 M sucrose. Conversion of both endogenously and exogenously labeled precursors was examined. For endogenous conversion experiments, secretory granules that had been isolated from previously radiolabeled tissue were incubated at 30°C for a specified period of time. For exogenous conversion experiments, unlabeled granule fractions were rapidly frozen and thawed six times to lyse the granules and were then incubated at 30°C with labeled prohormones obtained by gel filtration of extracts from tissue incubated separately with radiolabeled amino acids. Peptides recovered from the *M*_r 8,000–15,000 portion of the gel filtration fractionation range (prohormones, prohormone conversion intermediates, and minute amounts of nonhormonal proteins) were lyophilized, suspended in 0.04 M acetic acid, and then adjusted to pH 5.2 with 0.2 M sodium acetate before incubation with unlabeled granule preparations. The incubation procedure was established after performing experiments to determine the pH and temperature optima for conversion in isolated granules and experiments to compare the time-course of conversion in intact tissue and isolated granules. Anglerfish are isothermic at 4°–10°C. Incubation of intact tissue at temperatures above 20°C inhibits protein synthesis. The time-course of proinsulin and proglucagon conversion in intact tissue incubated at 20°C was paralleled closely by conversion in granules incubated at 30°C, with insulin and glucagon accumulation reaching a plateau between 3 and 6 h of incubation. Intact or lysed secretory granules were therefore incubated for 6–8 h at 30°C to assure that the conversion reactions had sufficient time to reach completion.

To examine the effects of various proteinase inhibitors on conversion, the two granule fractions isolated from labeled tissue were combined, diluted in 0.25 M sucrose, and precipitated by centrifugation. The granules were suspended in sodium acetate buffer (pH 5.2) and then frozen and thawed six times. Aliquots of the lysed granule preparations were then either extracted immediately or incubated for 6 h at 30°C in the presence or absence of specific inhibitors. All inhibitors were prepared just before incubation with the granules.

Extraction Procedure and Gel Filtration

Islet peptides were extracted with 2 M acetic acid as described by Noe et al. (13). Extracts were filtered on columns (1.6 cm × 90 cm) of Bio-Gel P-10 (100–200 mesh) in 2 M acetic acid at ambient temperature and 1.5-ml fractions were collected. Monitoring of the eluate for absorbance at 280 nm, determination of the distribution of radioactivity, and computation of double-label data were all performed as previously described (14). Copies of both the eluate chromatograms and the normalized data were made by use of a Tektronix hard-copy unit and retained for use in calculating hormone accumulation.

Determination of Percent Conversion

Evaluation of conversion of proglucagon and proinsulin was based on differential labeling of islet peptides. Tryptophan is incorporated into anglerfish proglucagon, glucagon, prosomatostatin, and somatostatin but not into proinsulin (10, 13–15). Percent accumulation of glucagon was obtained by calculation of the ratio of ³H radioactivity in the *M*_r 2,500–6,000 portion of the gel filtration eluates to total post-void volume ³H radioactivity. It has been demonstrated previously that 81% of the [³H]tryptophan-induced disintegrations per minute (dpm) eluting in *M*_r 2,500–6,000 portion of the fractionation range can be attributed directly to labeled glucagon, monodesamidoglucagon and a *M*_r 4,900 glucagon immunoreactive peptide presumed to be a conversion intermediate between proglucagon and glucagon (7, 15). Total post-void volume ³H dpm were used rather than the ³H dpm that elute at *M*_r 11,000–13,500, because it is now known that prosomatostatin (*M*_r 13,328), which also contains tryptophan, coelutes with proglucagon under the conditions employed (10, 16). Thus, the relative contribution of each of these prohormones to the total ³H label eluting at *M*_r 11,000–13,500 cannot be readily determined. Finally, it would not be accurate to include void volume radioactivity because it has been concluded that the radioactive peptides eluting in the P-10 void volume are not involved in islet hormone or prohormone synthesis (17). Therefore, the expression of glucagon accumulation chosen was: % accumulation of glucagon = [(³H dpm *M*_r 2,500–6,000)/(total post-void volume ³H dpm)] × 100. As an example of total post-void volume dpm, the ³H dpm in fractions 38–120 in Fig. 2 were used. Although this computation method underestimates “% conversion” of proglucagon, comparison of % accumulation between control and experimental incubations remains valid because each was calculated using the same method.

Using [¹⁴C]isoleucine or reduced [³⁵S]cystine, anglerfish proinsulin and insulin are selectively labeled and clearly defined as separate ¹⁴C- or ³⁵S-labeled peaks in Bio-Gel P-10 filtration eluates; glucagon lacks both amino acids and somatostatin lacks isoleucine (13, 14). Percent accumulation of insulin was obtained by summation of the dpm in the proinsulin and insulin peaks, then use of the formula: % accumulation of insulin = [(¹⁴C or ³⁵S dpm insulin)/(¹⁴C or ³⁵S dpm proinsulin + insulin)] × 100.

In those experiments involving the use of proteinase inhibitors, results were

expressed as percent inhibition, according to the formula: % inhibition = $1 - [(\% \text{ accumulation in the test incubation}) / (\% \text{ accumulation in the control incubation})] \times 100$. Pre-labeled secretory granules were employed in the experiments testing the effects of proteinase inhibitors. To normalize the percent conversion in the test and control incubations to the "zero time" level, the percent accumulation of insulin and glucagon found in an extract of an aliquot of lysed granules not subjected to incubation was subtracted from accumulation in both control and inhibitor-containing postincubated samples before the percent inhibition calculation was performed. Results were subjected to a one-tailed Student's *t* test using zero (% inhibition) as the test statistic.

Polyacrylamide Gel Electrophoresis (PAGE)

Gel electrophoresis was performed on 1.5-mm slab gels of 10% polyacrylamide at alkaline pH by the method of Davis (18). After electrophoresis, gel tracks were excised, frozen, and sliced into 2-mm slices. These were eluted in 1.5 ml of 1 M propionic acid for 48 h. Aliquots were assayed for distribution of radioactivity or immunoreactive insulin (12) and glucagon (14) as previously described.

High-pressure Liquid Chromatography (HPLC)

The HPLC apparatus consisted of a Perkin-Elmer (P-E) Series 3B liquid chromatograph (Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.) linked to a P-E LC-75 spectrophotometer and a P-E Sigma 10 chromatography data station. Reverse phase HPLC was performed with a 0.46×15 cm Supelcosil LC-18 column (Supelco Inc., Bellefonte, Pa.), using 0.25 N triethylammonium phosphate, pH 3.0, containing 21% (vol/vol) acetonitrile (19). Samples were run at ambient temperature with a flow rate of 1.5 ml/min and a column pressure of 1,350 psi. After sample injection, the run was continued for 10 min at 21% (vol/vol) acetonitrile. At this point, a linear gradient which reached 30% (vol/vol) acetonitrile in 30 min was begun. A 5-min period at 30% CH_3CN , followed by a 5-min linear gradient to 60% CH_3CN , and a 10-min gradient returning to the initial conditions completed each chromatography run.

Enzyme and Protein Assays

5'-Nucleotidase (EC 3.1.3.5) was used as a marker enzyme for the plasma membrane. It was assayed according to the procedure of Dixon and Purdom (20). Activity units are micrograms of phosphate released per hour at 37°C. β -N-Acetylglucosaminidase (EC 3.2.1.30) was employed as a marker for lysosomes and was assayed according to Sellinger et al. (21) as modified by Unkeless et al. (22), using *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide as the substrate. Activity units are nanomoles of *p*-nitrophenol released per hour at 37°C.

Determination of protein content was made by the method of Bradford (23), using bovine gamma globulin standards.

Electron Microscopy

Secretory granules were sedimented by centrifugation (142,000 g, 30 min) and fixed for 1.5 h at 4°C in 1.5% (vol/vol) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.2. Granules were then postfixed in 2.0% (wt/vol) osmium tetroxide in the same buffer, dehydrated through a graded series of ethanol and propylene oxide, and embedded in Epon. Thin sections were cut with a Porter-Blum Sorvall MT-2 (Ivan Sorvall, Inc., Norwalk, Conn.), stained with uranyl acetate and lead citrate, and examined with a Philips 300 electron microscope.

RESULTS

Enzyme Activities of Subcellular Fractions

The distribution of lysosomal and plasma membrane enzyme markers recovered from the subcellular fractions is shown in Fig. 1. The nuclear and two mitochondrial fractions (F-II_T and F-II_M) contained 59.9% of the β -N-acetylglucosaminidase activity, with 37.6% present in the F-II_T fraction. The lowest amounts of this enzyme were found in the microsomes and secretory granules; in fact, the combined activities of the two secretory granule fractions (F-II_B and F-III_C) comprised only 9.9% of the total activity recovered, whereas the microsomes (F-III) contained 12.3%.

The nuclear (F-I), mitochondrial (F-II_T and F-II_M) and microsome (F-III) fractions were found to contain 81.7% of the 5'-nucleotidase activity. The nuclear fraction contained 28.4%

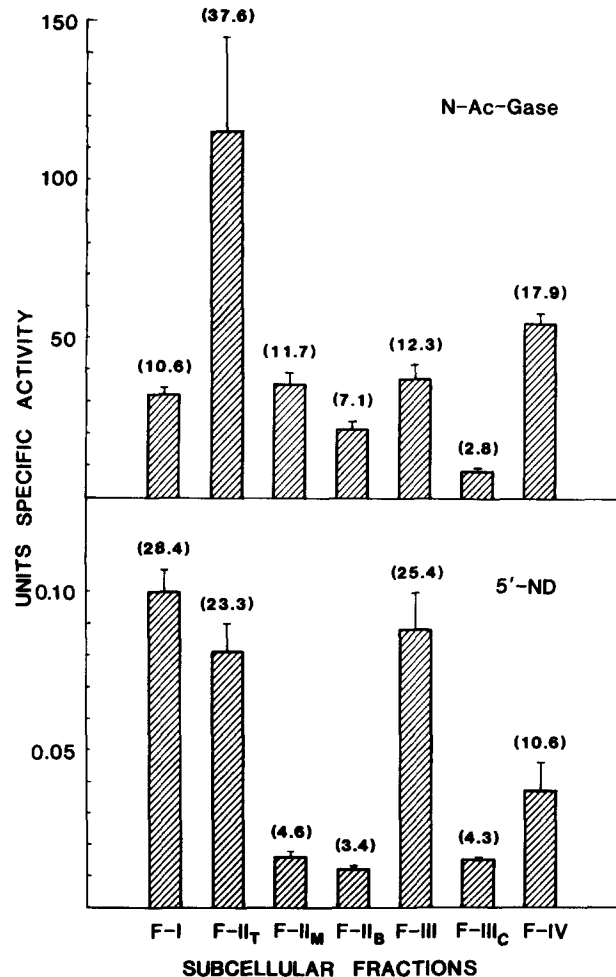


FIGURE 1 Subcellular distribution of enzyme activities. Aliquots were taken from the seven subcellular fractions and assayed for the presence of specific enzyme activities as described in Materials and Methods. Results are expressed as mean values (\pm SE) of specific activity (activity units of enzyme per milligram of protein) for six determinations. *N*-Ac-Gase, β -N-acetylglucosaminidase; 5'-ND, 5'-nucleotidase. Numbers in parentheses are percent of total activity recovered in each fraction.

of the total activity. This is attributed to the presence of unbroken cells in this fraction (12). The microsome fraction contained 25.4% of the recovered activity and the combined secretory granule fractions (F-II_B and F-III_C) contained only 7.7%. Together with the finding that most of the immunoreactive insulin, glucagon, and somatostatin is recovered in the granule fractions (10), these results demonstrate that contamination of the secretory granule preparation with other subcellular components is very low.

Determination of Optimum pH for Conversion

The data from determination of the optimum pH for conversion are shown in Figs. 2 and 3. Secretory granules were isolated from tissue incubated for 2 h with [³H]tryptophan and [¹⁴C]isoleucine and prepared for incubation as described in the legend of Fig. 2. After the granules were frozen and thawed, electron microscopic examination showed that ~95% of them were lysed (results not shown). In Fig. 2 B-D, the gel filtration elution patterns typically observed after incubation at three of the tested pH values are shown for comparison with the pattern obtained from granules extracted immediately after isolation

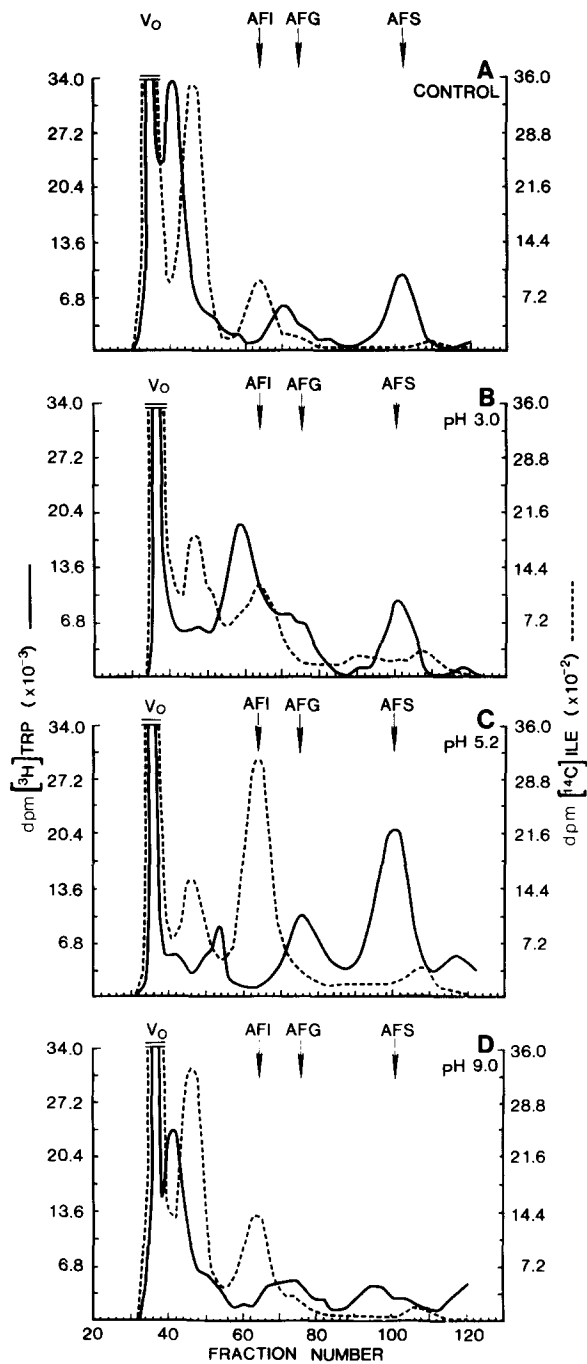


FIGURE 2 Effects of pH on islet prohormone conversion in secretory granules. Secretory granules were isolated from tissue incubated for 2 h with [^3H]tryptophan ([^3H]TRP) and [^{14}C]isoleucine ([^{14}C]ILE). Granules were suspended in 0.25 M sucrose, dispersed with homogenization by hand, and lysed by freezing and thawing six times. One aliquot was extracted immediately (nonincubated control). To remaining aliquots, an equal volume of 0.04 M sodium acetate-acetic acid buffer at pH 3.0, 4.0, or 5.2; or 0.04 M potassium phosphate buffer at pH 6.0, 7.5, or 9.0 was added and the preparations were incubated for 8 h at 30°C. Acetic acid extracts of each sample were subjected to gel filtration on Bio-Gel P-10 to determine insulin and glucagon accumulation at each pH value. A, Filtration pattern from extract of granules extracted immediately after isolation (nonincubated control); B, extract of granules incubated at pH 3.0; C, extract of granules incubated at pH 5.2; D, extract of granules incubated at pH 9.0; solid line, [^3H]tryptophan; dashed line, [^{14}C]isoleucine. Markers: Vo, void volume; AFI, AFG, AFS, elution positions of anglerfish insulin, glucagon, and somatostatin, respectively.

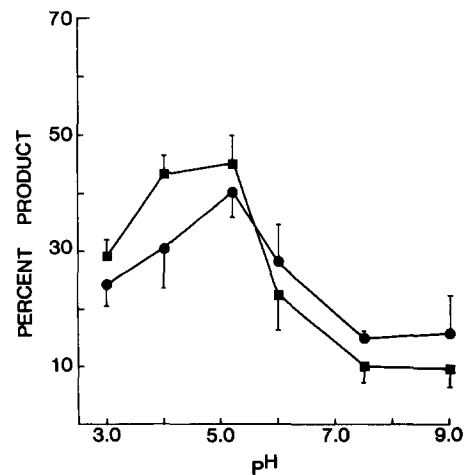


FIGURE 3 Determination of optimal pH for islet prohormone conversion in secretory granules. Secretory granules were isolated from labeled tissue, then resuspended and incubated as described in Fig. 2. After gel filtration of the resulting extracts, insulin and glucagon accumulation in excess of that found in nonincubated controls were determined as described in Materials and Methods and are expressed here as percent product. Data are mean (\pm SE) of two determinations. \bullet , Insulin; \blacksquare , glucagon.

(Fig. 2A). Prohormones and conversion intermediate(s) elute in the M_r 15,000–8,000 portion of the fractionation range (fractions 38–60). In Fig. 2, ^3H -labeled proglucagon and somatostatin elute near fraction 40 and ^{14}C -labeled proinsulin elutes near fraction 46 (1, 7, 10–17). The ^3H -labeled material that elutes near fraction 58 (Fig. 2B and C) indicates the presence of prohormone conversion intermediates (14, 17). As seen in Figs. 2B and D, accumulation of ^{14}C -labeled insulin, and ^3H -labeled glucagon and somatostatin (7, 10, 13, 14) was insignificant at pH 3.0 and pH 9.0. At pH 5.2, however, accumulation of all three hormones was pronounced (Fig. 2C). The elution patterns obtained at pH 5.2 were indistinguishable from those obtained from extracts of intact tissue (7, 10, 13, 14), or secretory granules isolated after pulse incubation and protected from lysis by suspension in 0.25 M sucrose during 6- to 8-h chase incubations. The data on hormone accumulation, obtained after incubation of lysed secretory granules in buffers at six different pH values, are summarized in Fig. 3. Conversion of proinsulin and proglucagon was found to be optimal between pH 4.5 and 5.5. The pH profile for somatostatin accumulation in the same experiments parallels the profiles shown in Fig. 3 (10).

By monitoring recovery of immunoassayable hormones in the media after incubation of freshly prepared granules in buffers at the pH values shown in Fig. 3, it was found that both α and β cell granules were most stable in the pH 4.5–5.5 range as well. Only 2.1% of the total immunoreactive insulin and 2.3% of the immunoreactive glucagon were found in the media after incubation at pH 5.2. The pH optimum for conversion and granule stability is significantly different from that for proteases in the cytosol and particulate fractions of anglerfish islet that degrade insulin and glucagon. Degradation of ^{125}I -insulin and ^{125}I -glucagon, monitored by loss of TCA-precipitable radioactivity, was found to be optimal at pH 3.4 and pH 7.3.¹ As shown in Fig. 2B, some prohormone degradation occurred, but little accumulation of insulin, glucagon, and somatostatin was observed after incubation at pH 3.0. In vitro

¹ G. E. Bauer, manuscript in preparation. Abstract: 1980, *Biol. Bull. (Woods Hole)*, 159:473.

conversion at pH 7.5 was even lower than that at pH 3 (Fig. 3). These results suggest that the precursor degradation observed at pH 3.0 and pH 7.5 was mediated to some extent by nonspecific or lysosomal proteases different from the converting enzyme(s).

Conversion of Exogenously Labeled Prohormones

It was assumed that labeled prohormones added to preparations of intact secretory granules would not readily pass through granule membranes to gain access to the converting enzyme(s). Indeed, conversion of labeled precursors added to unlysed secretory granules suspended in sodium acetate-buffered 0.25 M sucrose (pH 5.2) was found to be minimal; hormone accumulation was <5%. Because some of the proposed studies required the use of exogenously labeled precursors, it became necessary to determine: (a) whether unlabeled lysed granules could mediate exogenously labeled prohormone conversion, and (b) if conversion occurred, whether the "products" generated represented accurate cleavage products. In Fig. 4, prohormone conversion in granules isolated from labeled tissue (Fig. 4A) is compared with conversion of exogenously labeled precursors by an unlabeled lysed granule preparation (Fig. 4C). Fig. 4B shows the elution pattern of an extract prepared immediately after mixing labeled (exogenous) prohormones with lysed, unlabeled secretory granules. No radiolabeled hormones were present in the filtration eluate, indicating that neither nonspecific degradation nor conversion occurred during the preparation of the precursor peptides. When labeled prohormones were incubated for 6 h in buffer alone, the gel filtration pattern of the resulting extract was identical to that in Fig. 4B (not shown). When the lysed granule-prohormone mixtures were incubated for 6 h at 30°C, ¹⁴C-labeled insulin and ³H-labeled peptides having elution positions of glucagon-related peptides and somatostatin (10, 13–15) appear (Fig. 4C). Concomitant with the increased radiolabeling of the smaller peptides, there is a diminution of both ³H and ¹⁴C radioactivity in the precursor peptides. The elution patterns generated by granule-mediated conversion of both endogenously and exogenously labeled prohormones were indistinguishable from those obtained in extracts of intact tissue subjected to continuous or pulse-chase incubation (10, 13–15).

Three types of control experiment were performed. In the first two, labeled precursor peptides were incubated with rat liver homogenate or postmicrosomal supernate (F-IV) from anglerfish islet at pH 5.2. Although nearly complete degradation of labeled prohormones occurred in both types of incubation, accumulation of insulin, glucagon, or somatostatin was not observed in gel filtration eluates (data not shown). These results suggest that the prohormones and products were hydrolyzed by nonspecific proteases present in islet cytosol and rat liver homogenate. Incubation of labeled precursors with lysed islet secretory granules under the same conditions consistently yielded readily identifiable products (Fig. 4C). In the third type of control experiment, the fraction containing the greatest amount of lysosomal enzyme activity (F-II_T, Fig. 1) was tested for the ability to convert islet prohormones at pH 5.2. For these incubations the F-II_T protein:prohormone protein ratios were adjusted to one-half, equal to, and three times the secretory granule protein:prohormone protein ratios employed for the experiment in Fig. 4B and C. The F-II_T material was lysed by freezing and thawing, and the incubation conditions were the

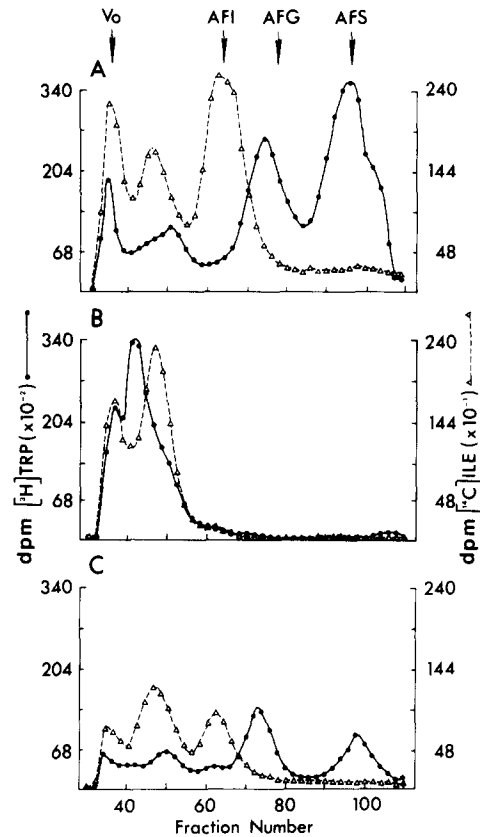


FIGURE 4 Conversion of radiolabeled prohormones to hormones by secretory granules. A, Secretory granules were isolated from islet tissue incubated for 3 h in the presence of [³H]tryptophan (³H-TRP) and [¹⁴C]isoleucine (¹⁴C-ILE). After suspension in 0.02 M sodium acetate buffer, pH 5.2, granules were incubated for 7 h at 30°C and then extracted with 2 M acetic acid. The distribution of radioactivity in the eluate after P-10 gel filtration is shown. B and C, Secretory granules isolated from unlabeled islet tissue were suspended in 0.02 M sodium acetate buffer, pH 5.2, then lysed by repeated freezing and thawing. Exogenously radiolabeled prohormones were obtained as described in Materials and Methods. Aliquots of the precursor preparation were combined with aliquots of the lysed granules and either extracted immediately with 2 M acetic acid (B) or allowed to postincubate for 6 h at 30°C (C) before extraction and gel filtration. ●, [³H]tryptophan; △, [¹⁴C]isoleucine. Markers: Vo, void volume; AFI, AFG, and AFS, elution positions of anglerfish insulin, glucagon, and somatostatin, respectively.

same as in Fig. 4C. Gel filtration analysis of the incubates showed that: (a) although ³H-labeled prohormones were degraded, proinsulin was very resistant to degradation, (b) the labeled "products" generated could not be identified as insulin, glucagon, or somatostatin on the basis of molecular size, and (c) the chromatographic profiles that resulted from incubations at all concentrations of F-II_T material were distinctly different from those seen in Figs. 2C and 4C, where conversion of endogenously and exogenously labeled prohormones, respectively, is shown.

Assessment of the Accuracy of Cleavage

To determine whether the products generated during *in vitro* conversion of exogenously labeled prohormones were accurate cleavage products (i.e., insulin- and glucagon-related peptides), the peptides eluting in the *M_r* 2,500–6,500 range of P-10 column filtrates were subjected to high-pH PAGE and reverse-phase HPLC. The electrophoretic mobilities of the products from

intact islet tissue are shown in Fig. 5A. The major ^{14}C -labeled component (fractions 16–19) was identified as anglerfish insulin, because it comigrated with the only detectable immunoreactive insulin eluted from the gel slices. Glucagon (fractions 8–11) and desamidoglucagons (fractions 21–26) were identified on the basis of tryptophan content and glucagon immunoreactivity. The 2 M acetic acid extraction and gel filtration procedures employed to recover somatostatin (10, 13, 17) promote selective deamidation of glucagon, which increases its electrophoretic mobility.² This accounts for the difference in the pattern shown in Fig. 5A as compared with those obtained when extraction is in acid ethanol and gel filtration is performed using 1 M acetic acid (14, 15). The enhanced mobility is in accordance with the results of Bromer et al. (24), who examined the electrophoretic mobility of deamidated glucagons at high pH using 20% acrylamide gels. Somatostatin is highly basic and migrates to the cathode under the conditions employed (10, 13, 17).

Labeled prohormones recovered from the same extract as the insulin and glucagon used in Fig. 5A were incubated 2 h in the presence of a lysed secretory granule preparation. Insulin- and glucagon-related peptides recovered from the P-10 filtrate of the resulting extract were run on the same slab gel as the products from intact tissue (Fig. 5B). The mobility of ^{14}C -insulin generated by granule-mediated cleavage of labeled prohormones was identical to insulin from intact tissue. Although it is apparent that the deamidated components predominate, the mobility of the glucagon-related peptides correlates precisely with those from intact tissue as well. It was found that increasing the duration of exposure of labeled prohormones to lysed secretory granules did not alter the electrophoretic mobility of insulin but promoted increased deamidation of glucagon. After 4 or 6 h of incubation, the primary [^3H]tryptophan-labeled glucagon immunoreactive component was the most rapidly migrating peptide (i.e., fractions 24–26). HPLC retention times of these products were consistent with the observed progressive deamidation of glucagon (data not shown).

The demonstration that the *in vitro* products cannot be distinguished from insulin- and glucagon-related peptides from intact tissue confirms that the secretory granule converting assay system provides a viable means for characterizing converting activity. In accordance with the results of Sorenson et al. (3), conversion of exogenous precursors was found to be less efficient than conversion of endogenous prohormones. This was probably because of substrate competition by unlabeled precursors in exogenous conversions in which the entire secretory granule content was unlabeled.

Converting Activity of Membranous and Soluble Components of Secretory Granules

To assess the relative converting activities of the membranous and soluble subfractions from granules, *in vitro* conversion assays were performed using exogenously labeled prohormones. Both membranous and soluble components of islet secretory granules were found to have significant prohormone converting activity (Table I). Both the membranous and the soluble converting activities were inhibited significantly by 300 $\mu\text{g}/\text{ml}$ leupeptin (see next section). To obtain a more accurate comparison of prohormone converting activity between the

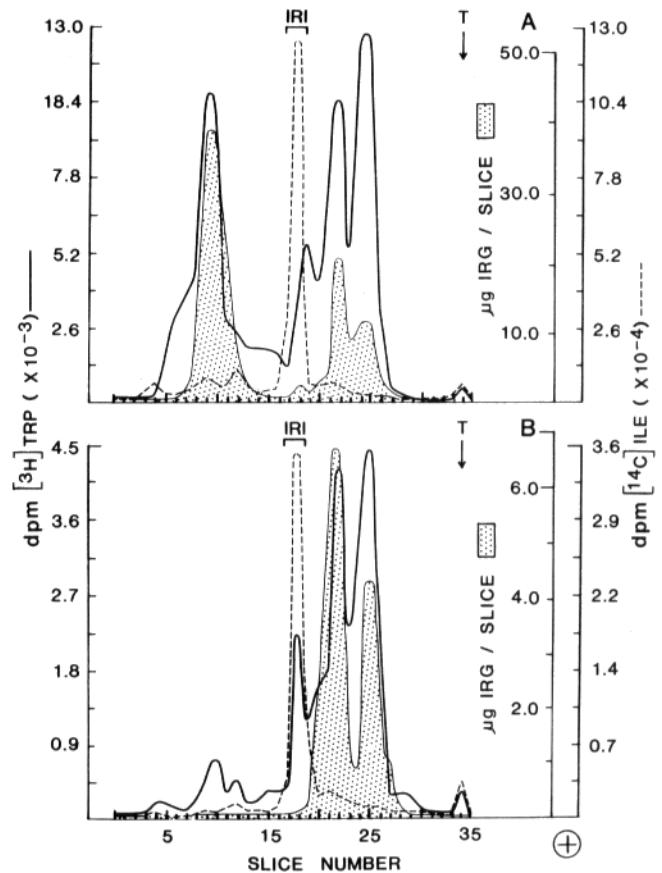


FIGURE 5 Comparison of the electrophoretic (polyacrylamide gel electrophoresis, PAGE) mobilities of insulin and glucagon extracted from intact islet tissue with products generated from conversion of prohormones mediated by lysed secretory granules. A, Labeled insulin- and glucagon-related peptides were recovered from the gel filtrate of an acetic acid extract of 73 mg of islet tissue incubated for 5 h with [^3H]tryptophan ([^3H]TRP) and [^{14}C]isoleucine ([^{14}C]ILE) and subjected to high pH PAGE. Gel slices were eluted and aliquots were taken for determination of the distribution of radioactivity and immunoreactive insulin and glucagon. Anglerfish insulin consistently eluted from slices 16–18. Glucagon and desamidoglucagons (see text and reference 24) eluted from slices 8–11 and 21–26, respectively. B, Labeled precursors were recovered from the same filtrate as in A above. The lyophilized prohormones were suspended as described in Materials and Methods and incubated at pH 5.2 for 2 h with aliquots of a lysed unlabeled secretory granule preparation. Acetic acid extracts were gel filtered and the insulin- and glucagon-containing portions of the eluate recovered for PAGE. The distribution of products generated by granule-mediated conversion coincides precisely with those extracted from intact tissue after a pulse incubation. Solid line, [^3H]tryptophan; dashed line, [^{14}C]isoleucine; stippled area, distribution of immunoreactive glucagon (right inner scale, $\mu\text{g}/\text{slice}$) brackets, areas in which single peaks containing 22.9 μg (A) and 4.6 μg (B) of immunoreactive insulin (IRI) were present; T, position of the tracking dye.

membranous and soluble components, “relative specific converting activity” was estimated by expressing percent accumulation of glucagon or insulin per milligram of membrane or supernate protein (Table I). When expressed in this manner, it appears that proinsulin was more readily converted than proglucagon by both the membranous and the soluble granule components in this *in vitro* system. It is most important to note, however, that even though the “specific converting activity” of the soluble subfraction was significantly greater than that of

² B. D. Noe, unpublished observations.

TABLE I
Conversion of Prohormones by Membranous and Soluble Components of Secretory Granules

Component	Percent accumulation*		Supernate‡ or membrane protein	Relative specific converting activity§	
	Glucagon	Insulin		Proglucagon	Proinsulin
Supernate	15.9 ± 3.9 (4)	34.3 ± 10.4 (4)	0.34 mg	46.7	100.8
Membrane	21.5 ± 4.0 (4)	36.6 ± 11.6 (4)	0.77 mg	27.9	47.5

Secretory granules from 60–80 mg of unlabeled tissue were isolated and lysed by freezing and thawing. Membranous components were separated from soluble components as described in Fig. 6. Each component was incubated with labeled prohormones for 6 h in equal volumes of buffer.

* Mean (± SE) percent accumulation determined as described in Materials and Methods for the number of observations in parentheses.

‡ Mean granule supernate and membrane protein assayed before incubation with exogenous prohormones. Prohormone content in all membrane and supernate incubations was equivalent.

§ Relative specific converting activity for proglucagon and proinsulin by membranous and soluble components was obtained by dividing the mean percent accumulation of product by the mean granule membrane or supernate protein content.

the membrane subfraction for both prohormones, the membrane subfraction clearly possesses some detectable converting activity.

However, it is possible that converting enzyme(s) may become nonspecifically adsorbed to the granule membranes after granule lysis. To further investigate this possibility, we subjected two identical granule membrane preparations to three successive washes in 0.04 M sodium acetate buffer, pH 5.2. For one of the membrane preparations, the second wash was performed in buffer brought to 100 mM KCl. This should promote the dissociation of adsorbed materials (see Discussion). After incubation of the two washed membrane preparations with labeled prohormones, the relative converting activities of the two preparations were compared. The accumulations of insulin and glucagon generated by the membrane preparation washed in KCl were only 16% and 5% lower, respectively, than those induced by the membranes washed in buffer only. These results suggest that a portion of the converting enzyme(s) in secretory granules is membrane associated.

Reverse-phase HPLC was used to analyze and compare the products of granule membrane- or supernate-mediated conversion (Fig. 6). Products generated from incubation with supernate (Fig. 6A) or membranes (Fig. 6B) were identical. The ¹⁴C label eluting at 42 min on HPLC was identified as insulin, based on coelution of immunoreactive insulin (Fig. 6B) and partially purified anglerfish insulin (Fig. 6C). Deamidated glucagons eluted at 28 and 32 min (Fig. 6A and B). Moreover, PAGE analysis of the products from both supernate- and precipitate-mediated conversion produced electropherograms identical to those obtained from nonfractionated lysed granule preparations (i.e., Fig. 5B).

Effects of Proteinase Inhibitors on Conversion

Islet tissue (150–200 mg) was incubated for 2.5–3 h in the presence of radiolabeled amino acids before fractionation. After lysing the secretory granules, we took one aliquot for immediate extraction and incubated the remaining aliquots for 6 h in the presence or absence of specific proteinase inhibitors. Leupeptin and antipain, which are arginine-containing peptide aldehydes (25), caused a significant reduction in both proglucagon and proinsulin converting activities (Table II). The thiol proteinase inhibitors 2,2'-dithiodipyridine (DDP) and *p*-chloromercuribenzoate (PCMB) also significantly inhibited both converting activities but did not alter the gel filtration elution position of either proglucagon or proinsulin. The inhibitory effects of PCMB and DDP were prevented when dithiothreitol (DTT), a reducing agent, was present in the incubations. Moderate inhibition of converting activity was also obtained

in the presence of *L*-tosylamide-2-phenylethylchloromethyl ketone (TPCK), an inhibitor of chymotrypsin. Inhibitors of serine proteinases (diisopropyl fluorophosphate [DFP], *p*-nitrophenyl-*p*'-guanidinobenzoate [NPGb], and soybean trypsin inhibitor [STI]) and metalloenzymes (EDTA) had no significant effect. Chloroquine and *N*-*p*-tosyl-*L*-lysine chloromethyl ketone HCl (TLCK), inhibitors of cathepsin B (26, 27), also failed to inhibit converting activity.

To determine whether the nontoxic inhibitors antipain and leupeptin could inhibit conversion in intact tissue, they were included in incubation media during 5-h incubations of islet tissue in the presence of radiolabeled amino acids. Both antipain and leupeptin caused an inhibition of prohormone-to-hormone conversion (Table III). The rate of protein synthesis was not affected by the inhibitors. The level of inhibition ranged from 22 to 25% when antipain or leupeptin was used singly at 500 μM. If antipain and leupeptin were combined at 500 μM each, inhibition of hormone accumulation was more effective. These results demonstrate that antipain and leupeptin can be assimilated and that these inhibitors interfere with prohormone-to-hormone conversion in intact islet tissue.

Competitive Effects of Natural and Artificial Substrates

Other than the fact that cleavage occurs at dibasic amino acid residues, there is currently very little information available regarding the enzyme-substrate interactions between converting enzyme(s) and prohormones. To test the hypothesis that a segment of polypeptide chain separate from the dibasic residue site may also be necessary for converting enzyme recognition of, and/or binding to prohormones, the relative abilities of unlabeled anglerfish islet precursors and an artificial substrate (porcine proinsulin) to inhibit labeled prohormone conversion were compared. In the absence of sufficient quantities of a purified anglerfish islet prohormone to use as a natural substrate, the precursor pool (*M_r* 8,000–15,000) that contains all of the islet prohormones was employed. However, for the purposes of these experiments, this is actually advantageous. All of the anglerfish islet prohormones are slightly larger in molecular size than porcine proinsulin (7, 13, 14, 16, 17). Therefore, 1 μg of porcine proinsulin should contain more dibasic residues (more molecules) for converting enzyme recognition than 1 μg of the anglerfish islet prohormone mixture.

Identical quantities (by weight) of artificial substrate, natural substrate (unlabeled), or bovine serum albumin (BSA) were included in incubations with lysed granules plus labeled precursors. BSA at 600 μg/ml had no effect on converting activity.

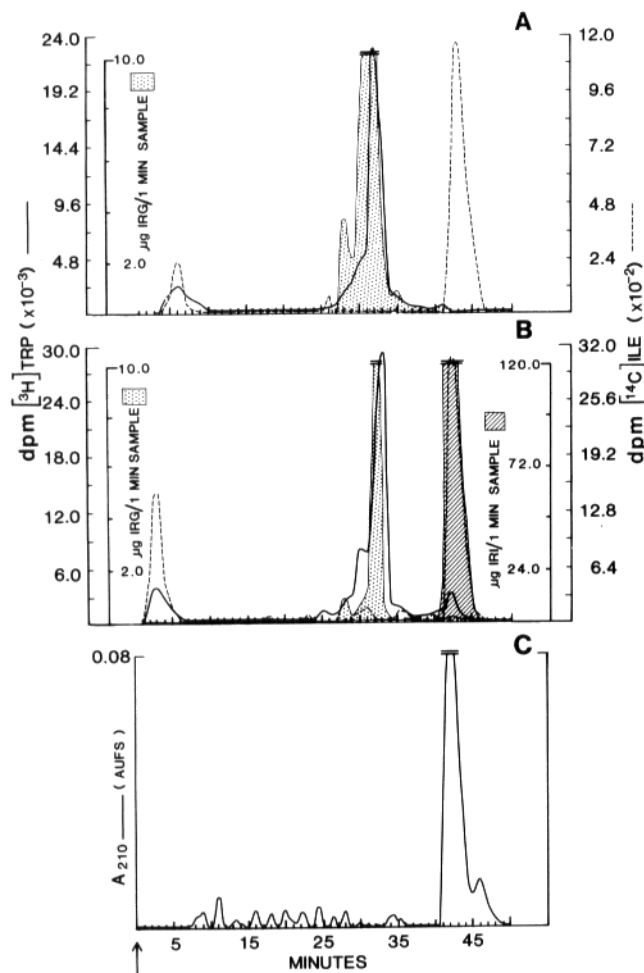


FIGURE 6 HPLC analysis of cleavage products generated by incubation of exogenously labeled prohormones with granule membranes and granule sol. Secretory granules prepared from 70 mg of islet tissue were suspended in 375 μ l of sodium acetate buffer (pH 5.2), dispersed with homogenization by hand, and then subjected to repeated freezing and thawing to disrupt granule membranes. Membranes were separated from soluble components of the granules by centrifugation of the lysate in a 2-ml tube for 30 min at 40,000 rpm using a Beckman 50 rotor. The supernate was removed and the membranes were washed by agitation in 100 μ l of acetate buffer and subjected to recentrifugation. After resuspension and dispersion of the granule membranes in a volume of sodium acetate buffer equal to that of the pooled supernates, aliquots were taken for protein determination; then each subfraction was incubated with an aliquot of the same labeled precursor preparation for 4 h. Acetic acid extracts of the incubates were subjected to gel filtration and the M_r 2,500–6,500 portion of the eluate was recovered, lyophilized, and suspended in 500 μ l of 3 M acetic acid for HPLC analysis. A, supernate; B, membranes; C, anglerfish insulin purified as described in reference 15. AUFS, absorbance units full scale; absorbance resulting from 500 μ l of 3 M acetic acid alone has been subtracted. Solid line, [3 H]tryptophan; [3 H]TRP dashed line, [14 C]isoleucine [14 C]ILE; stippled area, distribution of immunoreactive glucagon (left inner scales, μ g/1-min sample); cross-hatched area, distribution of immunoreactive insulin (right inner scale, μ g/1-min sample); arrow, sample injection mark.

The presence of porcine proinsulin in the incubation mixture inhibited accumulation of both glucagon and insulin (Fig. 7). However, for conversion of both proinsulin and proglucagon, the amount of inhibition caused by 600 μ g/ml unlabeled precursors was significantly greater than that caused by the

same amount of porcine proinsulin. The addition of 300 μ g/ml porcine proinsulin to an equal amount of natural substrate resulted in only slightly greater inhibition of both converting activities than that attained using 300 μ g/ml unlabeled precursors alone.

DISCUSSION

One potential difficulty in performing studies using isolated subcellular fractions is the possibility that the preparation employed is contaminated with particulate or soluble components from some portion of the subcellular apparatus other than the fraction of interest (28). For the purposes of the present study, it is important to know the level of contamination of the secretory granule fractions, especially with regard to lysosomal enzymes, because they could mediate prohormone degradation. As shown in Fig. 1, the combined secretory gran-

TABLE II
Effects of Proteinase Inhibitors on Prohormone Conversion by Secretory Granules

Inhibitor	Percent Inhibition	
	Proglucagon	Proinsulin
Antipain (500 μ M)	69.1 \pm 11.8* (3)	63.8 \pm 10.8* (3)
Chloroquine (100 μ M)	8.2 \pm 3.8 (2)	5.8 \pm 14.0 (2)
DDP (300 μ M)	53.9 \pm 16.8‡ (4)	50.8 \pm 17.3‡ (4)
DDP (300 μ M) + DTT (5 mM)	0.0 \pm 2.6 (2)	22.1 \pm 7.7 (2)
DFP (1 mM)	8.8 \pm 1.8 (2)	7.8 \pm 4.3 (3)
EDTA (1 mM)	-6.2 \pm 8.2 (2)	-26.0 \pm 21.6 (2)
Leupeptin (500 μ M)	49.2 \pm 5.5* (4)	47.5 \pm 12.4‡ (4)
NPGB (10 μ M)	-3.2 \pm 7.4 (2)	-1.4 \pm 5.6 (2)
PCMB (100 μ M)	93.4 \pm 3.4* (3)	56.4 \pm 2.6* (3)
PCMB (100 μ M) + DTT (5 mM)	-18.8 \pm 17.3 (4)	-27.8 \pm 34.9 (4)
PCMB (1 mM)	87.9 \pm 12.1* (3)	57.9 \pm 9.0 (3)
STI (25 μ M)	4.1 \pm 4.4 (3)	2.4 \pm 4.4 (3)
TLCK (200 μ M)	-4.8 \pm 14.5 (2)	-6.5 \pm 11.5 (2)
TPCK (200 μ M)	30.3 \pm 8.1 (2)	29.7 \pm 0.1* (2)

Secretory granules from labeled tissue were isolated, lysed by freezing and thawing, then incubated for 6 h in the presence or absence of inhibitors. Hormone accumulation and percent inhibition were calculated as described in Materials and Methods. Data represent mean values (\pm SE) for the number of observations in parentheses. The presence of a negative number signifies enhancement of conversion. The data were tested for significance using a one-tailed Student's *t* test with zero (percent inhibition) as the designated test statistic.

* $P \leq 0.01$.

‡ $P \leq 0.05$.

TABLE III
Effects of Antipain and Leupeptin on Prohormone Conversion in Intact Tissue

	Percent Inhibition*	
	Proglucagon	Proinsulin
500 μ M Antipain (AP)	25.0	22.1
500 μ M Leupeptin (LP)	22.4	23.1
500 μ M AP + LP	36.2 \pm 1.1	42.4 \pm 4.0

* Antipain and leupeptin were included in the media of tissue preincubated for 30 min, then incubated for 5 h with radiolabeled amino acids. Percent accumulation of insulin and glucagon were calculated after gel filtration of tissue extracts. Percent inhibition was calculated by comparing accumulation in experimental with insulin and glucagon accumulation in control incubations lacking the inhibitors (see Materials and Methods). Data are results from single experiments, except AP + LP at 500 μ M, where the data are mean (\pm SE) of two determinations.

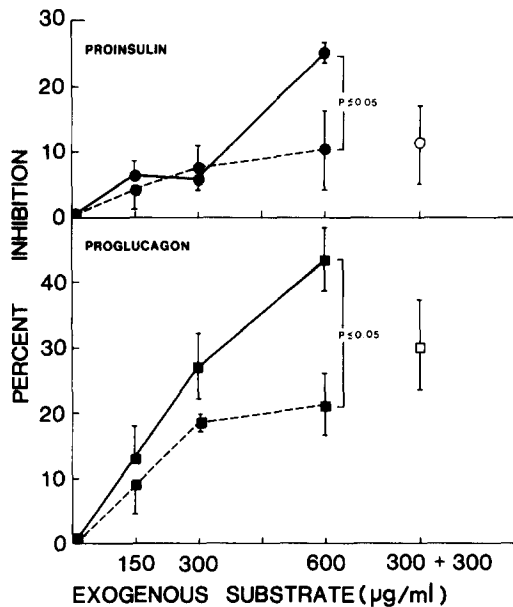


FIGURE 7 Effects of unlabeled precursor peptides and porcine proinsulin on conversion. Unlabeled anglerfish precursor peptides obtained by gel filtration of tissue extracts (solid line and solid symbols), or porcine proinsulin (dashed line and solid symbols), were added in concentrations of 150, 300, or 600 $\mu\text{g}/\text{ml}$ to aliquots of a lysed secretory granule-exogenously radiolabeled prohormone mixture. Equal amounts of each substrate (300 $\mu\text{g}/\text{ml}$) in the same incubation were also tested for inhibitory effects on proinsulin (\circ) and proglucagon (\square) conversion. Each combination was incubated for 6 h at 30°C before extraction. Insulin and glucagon accumulation were monitored in eluates after gel filtration of the extracts. Results are expressed as mean values (\pm SE) for three determinations. Bovine serum albumin at 600 $\mu\text{g}/\text{ml}$ had no inhibitory effect on prohormone conversion.

ule fractions were found to contain \sim 10% of the total *N*-acetylglucosaminidase activity, a low but significant level of contamination. However, the possibility that contaminating lysosomal enzymes are responsible for more than a minimal amount of granule-mediated degradation of labeled prohormones and hormones at pH 5.2 appears remote. Depletion of both endogenously labeled and exogenously labeled prohormones was accompanied by accumulation of identifiable hormones after incubation with lysed granules in acetate buffer at pH 5.2 (Figs. 2C, 4C, 5, and 6). This was also true for conversion of endogenously labeled precursors in intact granules (Fig. 4A). The fractions that contain the highest levels of lysosomal enzyme activity, F-II_T (38%) and F-IV (18%), although able to promote some protein degradation, were found to be incapable of mediating prohormone conversion when incubated under identical conditions. This was true for the fraction containing the most lysosomal enzyme activity (F-II_T) even when the fraction protein:prohormone ratio was three times that normally used for secretory granule incubations. These results strongly suggest that the product accumulation monitored in incubations performed at pH 5.2 in intact and lysed granules is primarily the result of secretory granule-associated converting enzyme activity and not contaminating lysosomal or cytosolic enzyme activity. The results from analysis of the products generated by granule-mediated conversion using PAGE (Fig. 5) and reverse-phase HPLC (Fig. 6) substantiate this conclusion. Each of the insulin- and glucagon-related products formed by granule-mediated conversion was found as a major component in extracts of whole tissue. In

view of the resolving power of HPLC for small peptides (19), it seems clear that lysed granules mediate accurate prohormone cleavage at pH 5.2.

The pH optimum for conversion of both proinsulin and proglucagon (Figs. 2 and 3), and prosomatostatin (10), was found to be in the pH 4.5–5.5 range. Sorenson et al. (3) reported that there were two pH optima for conversion of proinsulin in rat islets, pH 5.5 and 6.5. Previous reports on the pH stability of islet secretory granules (5, 29, 30) also correlate with our finding that granule stability was greatest in the pH 4.5–5.5 range as well. The pH range for optimum prohormone conversion differs significantly from the optima for insulin- and glucagon-degrading enzymes found in particulate and cytosolic fractions of anglerfish islet. This finding further emphasizes the distinct differences between prohormone converting activity and nonspecific degradation mediated by islet subcellular fractions. It is noteworthy that where internal pH has been determined in secretory granules isolated from other secretory tissues, it has been found to be near pH 5.5 (31, 32). It is possible that low intragranular pH is a characteristic of secretory granules from many tissues and that control of intragranular pH may play a role in regulation of physiologic events occurring within secretory granules such as prohormone conversion.

The integrity of the secretory granule membrane is not required for converting activity *in vitro*. Lysed granules were shown to mediate accurate cleavage in the present study (Figs. 4–6). Kemmler et al. (5) were unable to demonstrate conversion of exogenous proinsulin by lysed crude granule preparations from rat islets. Sorenson et al. (3) and Zühlke et al. (33), however, were able to demonstrate the conversion of exogenous proinsulin by granule preparations isolated from rat islets. Our findings are in accordance with the results from the latter two investigations on proinsulin conversion and demonstrate for the first time that exogenous proglucagon can be converted to glucagon by lysed secretory granule preparations.

The observation that proglucagon and proinsulin converting activities were present in the membranous components of secretory granules even after washing with 100 mM KCl, which should dissociate most adsorbed proteins (28), suggests that a significant proportion of the converting enzyme(s) is membrane bound. The activity detected in the granule supernates may be a normal constituent of the soluble components in the granule core. However, it seems more likely that this activity is derived from membrane-bound converting enzyme(s) that becomes detached or dislodged from the membranes upon repeated freeze-thawing. This supposition is supported by the finding that the proteolytic activities being monitored in membranous and soluble subfractions were very similar. Reverse-phase HPLC analysis of conversion products derived from exogenous precursors incubated with granule membranes or supernates demonstrated that the labeled cleavage products generated were identical (Fig. 6). Moreover, both the membranous and soluble converting activities were inhibited by leupeptin. Sun et al. (6), using secretory granules isolated from rat islet, found that only granule membranes, and not granule supernates, converted proinsulin to insulin. These results are consistent with the existence of a membrane-bound converting enzyme(s) in rat islet secretory granules as well.

The significant inhibition of the conversion of both proinsulin and proglucagon in the presence of antipain, leupeptin, DDP, or PCMB (Table II) indicates that the converting enzyme(s) is a thiol proteinase with a possible specificity for arginine residues. A similar pattern of susceptibility to these

inhibitors was found for prosomatostatin conversion (10). That antipain and leupeptin were capable of inhibiting conversion in the intact cell (Table III) supports the argument that the conversion monitored in lysed granule preparations represents the activity that occurs *in vivo*. The inhibition by DDP and PCMB was not attributable to the induction of a detectable conformational change of the precursor peptides, because the elution positions of proglucagon and proinsulin were unchanged in the presence of either inhibitor. Furthermore, the reversal of the inhibitory action of DDP and PCMB by DTT demonstrates the participation of a reactive sulfhydryl group in the converting enzyme. Reactive sulfhydryl groups are characteristic of thiol proteinases (34). Kemmler et al. (5) found that conversion of rat proinsulin by a crude granule fraction was inhibited in the presence of PCMB, suggesting the involvement of a thiol proteinase in rat islet as well. The moderate amount of inhibition of prohormone conversion caused by TPCK is consistent with previous findings that this compound is also highly reactive with thiol groups (35). The possible specificity of the converting enzyme(s) for arginine residues is suggested by the inhibition of conversion in the presence of antipain and leupeptin. These peptides contain an arginine aldehyde that is essential for inhibition (25). There was no significant reduction of the proglucagon or proinsulin conversion in the presence of inhibitors of metalloenzymes or serine proteinases. DFP, the most potent serine proteinase inhibitor, did not inhibit conversion significantly even though the concentration used was in excess of that sufficient to inhibit milligram quantities of trypsin.

Because pairs of basic amino acid residues have been found at cleavage sites in most polypeptide prohormones, the involvement of enzymes with specificity for basic residues in the processing of proinsulin has been suggested by a number of investigators. Proposed converting enzymes include "trypsin-like" enzymes (5, 33, 36), cathepsin B (37, 38), pancreatic kallikrein kinase (39), and plasminogen activator-plasmin (40). The role of enzymes with similar specificity in the conversion of anglerfish islet prohormones has been demonstrated by investigations in which trypsin treatment of anglerfish proglucagon (15, 41) and proinsulin (15, 42) yielded peptides having some of the characteristics of glucagon and insulin, respectively. However, on the basis of the proteinase inhibitor data presented in this paper and in a previous paper (10), it is concluded that none of the converting enzymes that have been implicated previously is involved in the conversion of anglerfish prohormones by secretory granules. Both kallikrein (43) and plasminogen activator (44) are inhibited in the presence of DFP and NPGB, neither of which affected conversion of anglerfish prohormones. The involvement of trypsin *per se* is also negated by the failure of DFP, STI, and TLCK to inhibit conversion. In view of the pH optimum for conversion and the susceptibility of the converting process to thiol proteinase inhibitors, the possibility arises that the anglerfish converting enzyme(s) may be cathepsin B. This lysosomal thiol proteinase is susceptible to inhibition by PCMB, antipain, and leupeptin and has its greatest enzymatic activity at pH 3.5–6.0 (26, 27). Moreover, the results from two recent studies have demonstrated that cathepsin B catalyzed the conversion of proinsulin into insulinlike components (37, 38). In lysed anglerfish islet secretory granules, however, neither proglucagon nor proinsulin conversion was inhibited in the presence of chloroquine or TLCK, both of which are powerful inhibitors of cathepsin B (26, 27). These data indicate that the converting activity ob-

served is mediated by an enzyme other than cathepsin B, but which has cleavage specificity and a pH optimum similar to those of cathepsin B.

The data from the present study do not provide any information regarding the possibility that a two-step cleavage process may be involved in anglerfish islet prohormone conversion. It has been demonstrated for several polypeptide prohormones that the combined action of trypsin and carboxypeptidase B are required to effect complete prohormone-to-hormone cleavage *in vitro* (45–47). Because of its primary structure, anglerfish proinsulin can be converted to insulin with single cleavages at either end of the C-peptide by a trypsinlike enzyme alone (42, 48). Although mammalian "proglucagons" (46) and glicentin (49) require a two-step cleavage mechanism, the primary structure of anglerfish proglucagon is unknown. It is therefore not possible to state whether the inhibitors that interfered with proglucagon converting activity were inhibiting the action of one or more than one enzyme.

It is proposed that the converting enzyme(s) in the anglerfish islet is a unique intracellular proteinase that may be granule membrane-associated and has the characteristics of a thiol proteinase with cleavage specificity for basic amino acid residues. Because conversion of the prohormones is also detected in microsome fractions (7, 10, 13), it is conceivable that the converting enzyme(s) is synthesized along with the (pre)prohormones in the rough endoplasmic reticulum and becomes associated with membranous components during or soon after synthesis. The results from the experiments in which the ability of porcine proinsulin to inhibit exogenous conversion was assessed are consistent with the possibility that the converting enzyme(s) requires the presence of a segment of polypeptide in addition to the dibasic residue sites for recognition and/or binding to the prohormones (Fig. 7). Porcine proinsulin was found to be significantly less potent as an inhibitor of conversion than equal microgram amounts of natural substrates. Because they are all larger than porcine proinsulin, the mix of natural substrates provided fewer dibasic residue sites (fewer substrate molecules) for enzyme action in these experiments. Because porcine proinsulin, which presented more dibasic residue-containing molecules to the system, competed less well with labeled natural substrates than the unlabeled natural substrates, the results suggest that the converting enzyme(s) recognizes and/or requires more than just dibasic residues for performing its function.

It remains to be determined whether there are separate converting enzymes for each islet prohormone. If there are separate converting enzymes for each islet prohormone, then each has the same sensitivity to proteinase inhibitors (Table II) and a similar pH optimum (Figs. 2 and 3; reference 10). To completely resolve this and other questions regarding the subcellular conversion of anglerfish proglucagon and proinsulin, it will be necessary to isolate and characterize the converting enzyme(s).

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