

# Comparison of Prohormone-processing Activities in Islet Microsomes and Secretory Granules: Evidence for Distinct Converting Enzymes for Separate Islet Prosomatostatins

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**ABSTRACT** In previous work we have examined the nature of converting enzymes for proinsulin, proglucagon, and prosomatostatin-I (PSS-I) in secretory granules isolated from anglerfish islets. The purpose of the present study was to extend the examination of precursor conversion to islet microsomes and to compare prohormone processing, including that of PSS-I and prosomatostatin-II (PSS-II), in islet secretory granules and microsomes. Microsomes (rough endoplasmic reticulum [RER] and Golgi complex) and secretory granules were prepared from anglerfish islets by differential and discontinuous density-gradient centrifugation. Microsomes were further fractionated into Golgi- and RER-enriched subfractions. Lysed secretory granule or microsome preparations were incubated in the presence of a mixture of radioactively labeled islet prohormones. Extracts of products generated were subjected to analysis by gel filtration and high-pressure liquid chromatography. Accuracy of product cleavage was monitored by comparing high-pressure liquid chromatography retention times from the radiolabeled *in vitro* conversion products with the retention times of labeled products from tissue extracts.

All converting activity in microsomes was found to be similar to that in granules in that it had a pH optimum near pH 5 and was inhibited by *p*-chloromercuribenzoate. No significant differences in the converting activity of Golgi complex- and RER-enriched subfractions of microsomes was observed. The proinsulin, proglucagon, and PSS-II converting-enzymes, which were found in islet secretory granules, were also present and membrane-associated in islet microsomes. However, converting activity for PSS-I was displayed only in secretory granules. This suggests that two or more separate enzymes are involved in processing PSS-I and PSS-II, and that these enzymes have either differential distribution or differential activity in RER/Golgi complex and secretory granules. The demonstration of converting enzyme activity in islet microsomes supports the proposal that these enzymes may be synthesized at the RER and are internalized along with the prohormones.

Although there is a large body of evidence that demonstrates that most peptide hormones destined for export from their cells of origin are synthesized initially as preprohormones, very little is known about the subcellular distribution and characteristics of the enzymes involved in co- and posttranslational proteolytic processing of these precursors. In previous studies performed in this laboratory, the characteristics of the converting activities of proinsulin, proglucagon, and proso-

matostatin-I found in secretory granules from anglerfish (AF)<sup>1</sup> pancreatic islets were examined. It was found that one or

<sup>1</sup> *Abbreviations used in this paper:* AF, anglerfish; HPLC, high-pressure liquid chromatography (reverse phase); PSS-I and II, anglerfish prosomatostatin-I and II; RER, rough endoplasmic reticulum; RT, retention time; SS-14, anglerfish/synthetic somatostatin-14 (cleavage product of PSS-I); SS-28, anglerfish/somatostatin-28 (cleavage product of PSS-II).

more of the enzymes involved in mediating the conversion of these prohormones had unique characteristics that differentiate them from other intracellular proteases. The enzyme(s) is a cysteine proteinase, has a pH optimum near 5, cleaves as an endoprotease at basic amino acid residues, may require the presence of segments of the prohormone in addition to the dibasic residues at cleavage sites for substrate recognition and/or binding, and is (secretory granule) membrane associated (1-3). The enzyme(s) has some of the characteristics of cathepsin B but differs from cathepsin B in that it is not inhibited by *N*-*p*-tosyl-L-lysine-chloromethyl ketone HCl and has a more restricted substrate specificity. Converting enzymes cleave more selectively than cathepsin B, usually at sites where pairs of basic amino acids are found in the polypeptide sequence. However, not all basic pairs that are found in prohormones serve as cleavage sites.

It would be consistent with the data presently available to hypothesize that the acid cysteine proteinase found in secretory granules from AF islets performs the initial "trypsinlike" cleavage, which was originally proposed as the first processing step necessary in proinsulin conversion (4-9), and is necessary in the processing of most other prohormones. Recently, prohormone-converting enzymes with characteristics very similar to the acid cysteine proteinase in AF islet granules have been identified in secretory granules from rat pituitary anterior lobe (10), neurointermediate lobe (11), and from bovine pituitary posterior lobe (12).

In addition to proinsulin and proglucagon(s), at least two different forms of somatostatin-like peptide are produced in AF islets. These include prosomatostatin-I (PSS-I), the precursor of anglerfish/synthetic somatostatin-14 (SS-14), and a second precursor (PSS-II), which contains at its COOH-terminus [Tyr<sup>7</sup>, Gly<sup>10</sup>] SS-14 as a potential cleavage product. However, even though an Arg-Lys pair is located immediately NH<sub>2</sub>-terminal to Ala<sup>1</sup> of the COOH-terminal tetradecapeptide, [Tyr<sup>7</sup>, Gly<sup>10</sup>] SS-14 could not be detected in extracts of AF islets (13, 14). Subsequently, a 28-residue peptide (AF SS-28) that has [Tyr<sup>7</sup>, Gly<sup>10</sup>] SS-14 as its COOH-terminus was found to be a primary cleavage product of PSS-II (manuscript in preparation). The purpose of the present study was to compare the converting activity(ies) of prohormones in microsomes with those in secretory granules and to examine the differential processing of the two somatostatin precursors in more detail. Experiments were designed to determine; (a) whether the processing activities of proinsulin, proglucagon, PSS-I, and PSS-II could be found in both islet microsomes and secretory granules; (b) whether the processing activities were membrane-associated or soluble in these fractions; and (c) whether there was a difference in the relative contribution of Golgi complex and rough endoplasmic reticulum (RER) to overall converting activity of microsomes.

## MATERIALS AND METHODS

**Materials:** L-[<sup>3</sup>H]tryptophan (8 Ci/mmol) and L-[<sup>35</sup>S]cysteine (928 Ci/mmol) were purchased from New England Nuclear, Boston, MA. L-[<sup>14</sup>C]isoleucine (300 mCi/mmol) was purchased from ICN Pharmaceuticals, Irvine, CA. Bio-Gel P-2 (100-200 mesh), P-30 (50-100 mesh and 100-200 mesh), and Protein Reagent Kit were obtained from Bio-Rad Laboratories, Rockville Center, NY. Monofluor scintillation cocktail was purchased from National Diagnostics, Inc., Somerville, NJ. High-pressure liquid chromatography (HPLC) grade acetonitrile and 2-propanol were obtained from Fisher Scientific Co., Atlanta, GA, and reagent grade trifluoroacetic acid used in the HPLC solvents was purchased from Pierce Chemical Co., Rockford, IL. Cocarboxylase (thiamine pyrophosphate), phosphorus standard, acid molybdate, and Fiske

and Subbarow reducer were obtained from Sigma Chemical Co., St. Louis, MO. Synthetic SS-14 and [Tyr<sup>7</sup>, Gly<sup>10</sup>] SS-14 were the generous gifts of Dr. Jean Rivier, Salk Institute, San Diego, CA.

**Preparation of Microsomes and Secretory Granules:** Fractionation of decapsulated islet tissue was performed as previously described (2, 15) with all procedures being carried out at 4°C. For the present studies, the two secretory granule fractions (granule diameter ranging [a] from 150 to 200 nm and [b] from 220 to 310 nm) were pooled, diluted in 0.25 M sucrose and 1 mM MgCl<sub>2</sub>, and precipitated at 150,000 *g* for 30 min. The F-III band (microsomes) was aspirated from the surface of the 1.4 M sucrose, transferred to another centrifuge tube, and also precipitated at 150,000 *g* for 30 min. As previously determined, these fractions show very little contamination by lysosomal enzymes (2). For conversion assays, the microsome and granule pellets were suspended separately in 100 mM sodium acetate-acetic acid, pH 5.2 and the particulates were dispersed by using a hand-held Teflon pestle-glass homogenizer. Before incubation with labeled prohormones of *M*<sub>r</sub> 8,000-15,000 (see below), the dispersed secretory granule and microsome suspensions were lysed by six cycles of rapid freezing and thawing. For experiments designed to assess the converting activity of the soluble and membranous components of microsomes or secretory granules independently, lysed microsomes or granule preparations were subjected to centrifugation at 12,800 *g* for 10 min at 4°C in a microcentrifuge. Supernatants were removed and saved; membranous precipitates were washed in 100 mM sodium acetate-acetic acid, pH 5.2, and reprecipitated twice. The resulting pellet was suspended in 500 μl of the same buffer and transferred to a hand-held homogenizer to disperse the membranes. For some experiments, membranes were washed with 1 M KCl to remove membrane-associated proteins before performance of conversion assays. In these instances membrane preparations were suspended and dispersed in 100 mM sodium acetate-acetic acid, 1 M KCl, pH 5.2. After 30 min at 4°C, membranes were precipitated by a 10-min centrifugation in the microcentrifuge and washed twice with 100 mM sodium acetate buffer before final suspension for the conversion assay. For experiments in which microsomes were to be incubated at varying pH values, precipitates were suspended in 100 mM sodium acetate-acetic acid or 100 mM sodium phosphate at the appropriate pH. Aliquots were removed from all granule or microsome preparations to determine protein content.

**Preparation of Microsomal Subfractions:** To prepare Golgi complex- and RER-enriched subfractions of microsomes, centrifugation through a discontinuous sucrose density gradient was performed. Precipitated microsomes were suspended in 0.5 ml of 0.25 M sucrose and 1 mM MgCl<sub>2</sub> and dispersed in a hand-held homogenizer. The original centrifuge tube and homogenizer were washed twice with an additional 0.5 ml of sucrose-MgCl<sub>2</sub>. The discontinuous gradient was formed in a 5-ml cellulose nitrate tube (Beckman Instruments, Inc., Fullerton, CA) by sequentially layering over 0.5 ml of 2.0 M sucrose, 0.7-ml aliquots of 1.5, 1.4, 1.3, 1.2, and 1.1 M sucrose (all containing 1 mM MgCl<sub>2</sub>). The dispersed microsome preparation was then layered above the 1.1 M sucrose band and the tube was filled with 0.25 M sucrose, 1 mM MgCl<sub>2</sub>. Centrifugation was performed in an SW 50.1 rotor (Beckman Instruments, Inc.) at 142,000 *g* for 2 h. After centrifugation, four clearly visible particulate bands (1-2 mm wide) were observed at the interfaces between 1.1-1.2 M, 1.2-1.3 M, 1.3-1.4 M, and 1.4-1.5 M sucrose layers. Usually, a less distinct particulate band was found at the 1.5-2.0 M interface as well. These bands were designated subfractions 1-5, respectively. All bands were removed separately by aspiration, diluted in 0.25 M sucrose-1 mM MgCl<sub>2</sub>, and concentrated by centrifugation.

**Preparation of Labeled Prohormones:** Incubations of 75-100 mg of islet tissue were performed in the presence of 50 μCi [<sup>3</sup>H]tryptophan plus either 10 μCi [<sup>14</sup>C]isoleucine or 40 μCi [<sup>35</sup>S]cysteine as previously described (1-3). After a 5-h incubation with the labeled amino acids, the medium was removed and the tissue homogenized in 1.0 ml of 2 M acetic acid. Insoluble material was removed by centrifugation and reextracted with 0.5 ml of 2 M acetic acid. After centrifugation, the soluble components were pooled and desalted on columns of Bio-Gel P-2 in 2 M acetic acid and lyophilized. Desalted extracts were suspended in 2 M acetic acid and filtered in 2 M acetic acid on 1.6 × 96-cm columns of Bio-Gel P-30 (55% 50-100 mesh, 45% 100-200 mesh). The polypeptides of *M*<sub>r</sub> 8,000-15,000 were recovered by pooling and lyophilizing the appropriate portions of the gel filtration eluates. This pool contains AF proinsulin, proglucagon(s), and prosomatostatin(s) (1-3, 13, 14). Before incubation with lysed microsome or secretory granule suspensions, the precursors were suspended in 100 mM acetic acid and the pH was adjusted to 5.0 using 2 M sodium acetate. Aliquots were taken to determine protein content.

**In Vitro Conversion Assays:** Aliquots of lysed microsome or secretory granule suspensions, or aliquots of membranous or soluble components from these lysates, were combined with a fraction of the prohormone preparation and sufficient 100 mM sodium acetate-acetic acid, pH 5.2, (or other buffer when indicated) to bring the incubation volume to 1 ml. The membrane protein/prohormone protein ratios in the incubates ranged between 1:1 and

10:1. Before incubation, 1- $\mu$ l samples were removed to check the pH of the incubation medium using narrow range (pH 3.0–5.5) pH paper. If necessary, the pH was adjusted with either 2 M acetic acid or 2 M sodium acetate to be as close to 5.2 as possible. Incubations proceeded in capped siliconized glass vials for 6 h at 30°C in a metabolic shaking incubator. At the end of the incubation, 250  $\mu$ l of 10 M acetic acid was added to bring the final acetic acid concentration to 2 M. After a 30-min extraction period, insoluble material was removed by centrifugation and reextracted with 0.5 ml of 2 M acetic acid. The insoluble material was again removed and the pooled supernatant was subjected to gel filtration.

**Assay of Products:** Monitoring the accuracy of in vitro secretory granule or microsome-mediated prohormone conversion was accomplished by subjecting cleavage products recovered from gel filtration eluates to reverse-phase HPLC. The HPLC system consisted of a Perkin-Elmer series 3B liquid chromatograph (Perkin-Elmer Corp., Norwalk, CT) linked to a Perkin-Elmer LC-75 spectrophotometer and a Perkin-Elmer Sigma 10 chromatography data station. The columns employed were 0.46  $\times$  25 cm Vydac C<sub>18</sub> (300- $\text{Å}$  pore size, 5- $\mu$ m bead size; Separations Group; Hesperia, CA) columns end-capped with C<sub>2</sub>. Elution was accomplished by using a variable mixture of solutions A and B. Solution A was 0.24% trifluoroacetic acid (40%) and CH<sub>3</sub>CN (60%). Solution B was 0.1% trifluoroacetic acid. Acetonitrile concentrations during gradient elution are shown on each figure. Prohormone cleavage products were recovered from the *M<sub>r</sub>* 4,500–8,000 (insulin), *M<sub>r</sub>* 2,500–4,500 (glucagon-related peptides and SS-28), and the *M<sub>r</sub>* 1,000–2,000 (SS-14) portions of gel filtration eluates, lyophilized, suspended in freshly prepared 3 M acetic acid, and filtered through 0.22  $\mu$ m filters (Millipore Corp., Bedford, MA) before injection for HPLC. Accuracy of product cleavage was monitored by comparing the HPLC retention times of radiolabeled in vitro conversion products with those of labeled products from tissue extracts and, in the case of SS-14, with synthetic SS-14. As it is known that even slight modifications in peptide structure can significantly alter HPLC retention time (16), cleavage products generated in vitro that had HPLC retention times identical to those of tissue extract products were considered to be accurately cleaved. To compensate for slight variations in retention times of identifiable peptides which were observed periodically, a tissue extract control sample was always run on the same day as any conversion products to which its elution pattern was to be compared.

**Protein Assay, Radioimmunoassay, and Thiamine Pyrophosphatase Assay:** Protein was assayed according to the method of Bradford (17) and somatostatin radioimmunoassay was performed as previously described (18) using the centrally directed R141 antiserum. Thiamine pyrophosphatase was assayed by the method of Novikoff and Heuss (19) as modified by Meldolesi et al. (20).

**Electron Microscopy:** Microsomal subfractions were sedimented by centrifugation 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. After postfixation in 2.0% (wt/vol) OsO<sub>4</sub> in the same buffer, samples were dehydrated through a graded series of ethanol and propylene oxide and embedded in Epon. Thin sections were cut, mounted on Formvar-coated grids, stained with uranyl acetate and lead citrate, and examined with a Philips 400 electron microscope (Philips Electronic Instruments, Inc., Mahwah, NJ). Random photographs were taken of fields in sections from the center, intermediate, and surface areas of the pellet from each block.

## RESULTS

### Converting Activity of Secretory Granules

All results shown in Figs. 1–7 are representative examples of one from at least three experiments.

An example of the results from incubation of [<sup>3</sup>H]tryptophan and [<sup>14</sup>C]isoleucine-labeled prohormones with complete secretory granule lysates is shown in Fig. 1. Depletion of the prohormones of *M<sub>r</sub>* 8,000–15,000 and the appearance of smaller <sup>3</sup>H-labeled and <sup>14</sup>C-labeled peptides was observed. This pattern of label elution corresponds precisely with the patterns generated by gel filtration of extracts of pulse-incubated islet tissue (1–3, 13). Products from granule lysate-mediated conversions were recovered by pooling portions of gel filtration eluates, as indicated by the bars under Fig. 1B, to be examined by HPLC. Fig. 2, A–C depicts the HPLC elution pattern of labeled peptides of *M<sub>r</sub>* 1,000–2,000 generated by incubation of prohormones with complete granule lysate, granule lysate supernatant, and granule membranes,

respectively. The results demonstrate that the major [<sup>3</sup>H]-tryptophan-labeled product generated in all three types of in vitro incubation has a retention time identical with that of synthetic SS-14 (25 min). That this peptide was indeed somatostatin-related was determined by demonstrating that it possesses somatostatinlike immunoreactivity (Fig. 2A). The results in Fig. 2 indicate that both the soluble and membranous components of islet secretory granules contain an enzyme(s) capable of accurately cleaving PSS-I to yield SS-14.

A second [<sup>3</sup>H]tryptophan-labeled peptide from the *M<sub>r</sub>* 1,000–2,000 pool eluted with a retention time (RT) of 14 min. This is the elution position of [Tyr<sup>7</sup>, Gly<sup>10</sup>] SS-14 under the conditions employed. These results indicate that either PSS-II or AF SS-28 was cleaved to release [Tyr<sup>7</sup>, Gly<sup>10</sup>] SS-14 in these in vitro incubations. This, however, does not represent accurate cleavage of PSS-II, because [Tyr<sup>7</sup>, Gly<sup>10</sup>] SS-14 is not a normal cleavage product of PSS-II in the intact cell (13, 14). The implications of this observation are considered in the Discussion.

When the [<sup>3</sup>H]tryptophan plus [<sup>14</sup>C]isoleucine doubly la-

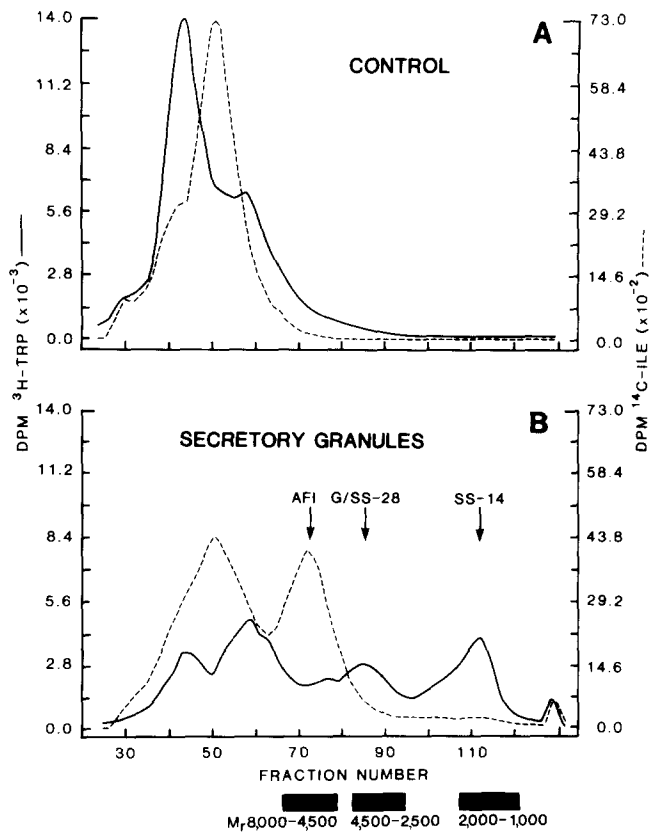


FIGURE 1 Islet prohormone conversion mediated by lysed secretory granules. [<sup>3</sup>H]tryptophan- and [<sup>14</sup>C]isoleucine-labeled islet polypeptides of *M<sub>r</sub>* = 8,000–15,000 (prohormones) were incubated 6 h in the absence or presence of a lysed secretory granule preparation. Granule lysate protein to substrate protein ratio was 4:1. Extracts were subjected to gel filtration as described in Materials and Methods. Alternate 1.5-ml fractions were assayed for radioactivity. The distribution of <sup>3</sup>H- (—) and <sup>14</sup>C- (----) radioactivity in the column eluates of the samples incubated in the absence and presence of a granule lysate is shown in A and B, respectively. Arrows in B indicate the elution positions of anglerfish insulin (AFI), glucagon-related peptides, and somatostatin-28 (G/SS-28), and somatostatin-14 (SS-14). The bars below B indicate regions of the eluate recovered for HPLC analyses.

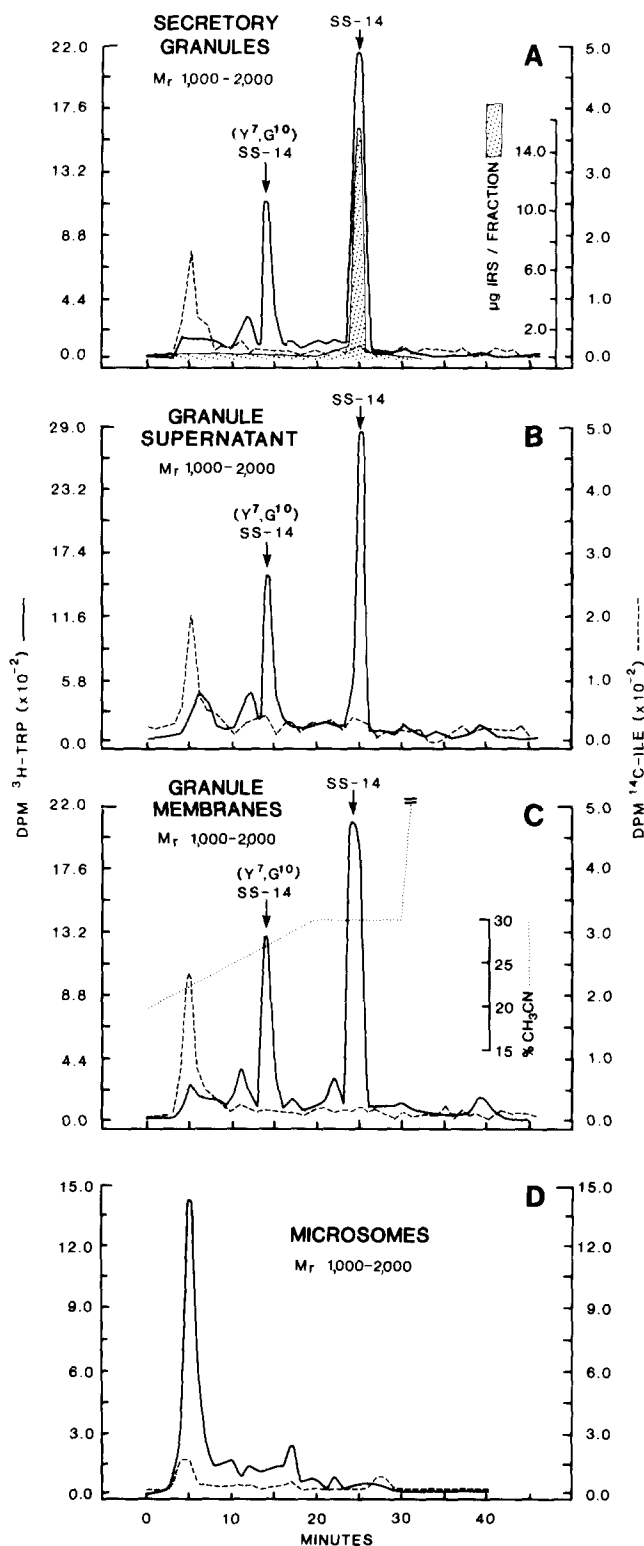


FIGURE 2 Action of granule and microsomal lysate components on PSS-I. Secretory granules were prepared from 173 mg of tissue and lysed. Two-thirds of the lysate was removed and separated into soluble and membranous components by centrifugation. Labeled prohormones of  $M_r = 8,000$ – $15,000$  were incubated 6 h with the remaining complete granule lysate, the soluble fraction, or with granule membranes. After gel filtration of the extracts, the peptides of  $M_r = 1,000$ – $2,000$  were recovered (bar, Fig. 1B) and aliquots were taken for HPLC analyses. Fractions of 0.8 ml (1.0 min) were collected to assay for radioactivity (A–C). The elution protocol was identical for each preparation (C). (D) Microsomes prepared from

beled peptides of  $M_r$  2,500–8,000 from tissue extracts were analyzed by HPLC.  $^3\text{H}$ -labeled AF SS-28 (Noe, B., and J. Spiess, manuscript in preparation) and  $^{14}\text{C}$ -labeled AF insulin (2) were clearly identified as products (Fig. 3). Products of proglucagon cleavage were also generated. All but one of the  $^3\text{H}$ -labeled peptides that appear in addition to SS-28 as eluate peaks in Fig. 3A have been identified by RIA as glucagon-related peptides (2 and unpublished results). Several of the glucagon-related peptides (RT 43.5, 45.5, and 47.0 min) were observed after granule-mediated conversion (Fig. 3B). Thus, the  $^3\text{H}$  labeling observed in the  $M_r$  2,500–8,000 region of gel filtration eluates (Fig. 1B) represents a combination of [ $^3\text{H}$ ]-tryptophan-labeled glucagon-related peptides and AF SS-28. When the granule supernatant and membrane components were examined separately for converting activity, it was found that both mediated the generation of insulin and some glucagon-related products; however, only the soluble component generated appreciable SS-28 (Fig. 4).

If the granule membrane preparations were washed with 1 M KCl (a treatment known to promote dissociation of membrane-associated proteins) before incubation, a significant reduction of granule membrane-mediated conversion resulted. KCl-washed membranes produced 69% less insulin, 55% less glucagon, and 47% less SS-14 than membranes washed in sodium acetate buffer alone. This suggests that a significant proportion of the enzyme(s) involved in converting several of the islet prohormones to their respective products is granule membrane-associated (Figs. 2C and 4C).

#### Converting Activity of Microsomes

Lysates of microsome fractions, when incubated with labeled prohormones at pH 5.2, were found to deplete the prohormones and to generate products with labeling patterns and molecular sizes similar to products derived from granule-mediated conversion (Fig. 5). Results from previous work have demonstrated that the pH optimum for *in vitro* conversion mediated by granule components is near pH 5.0 (2). To determine whether converting activity generated by microsomal components has a similar pH optimum, microsomal lysates were incubated with labeled prohormones in buffers at pH 5.2 and 7.5 as previously described (2). Microsome-mediated prohormone conversion proceeded readily at pH 5.2. Incubation at pH 7.5 completely abolished microsome-mediated conversion (data not shown). It has also been demonstrated previously that cysteine proteinase inhibitors block islet granule-mediated converting activity (1, 2). To determine whether a cysteine proteinase inhibitor was effective in inhibiting conversion by microsomes as well, the sensitivity of microsomal lysate-converting activity to *p*-chloromercuribenzoate was monitored. It was found that 100  $\mu\text{M}$  *p*-chloromercuribenzoate was a very effective inhibitor of prohormone conversion (data not shown). Therefore, several characteristics of converting-enzyme activity in microsomal lysates appear

58 mg of tissue were lysed and incubated with labeled prohormones; the material of  $M_r = 1,000$ – $2,000$  from the resulting extract was subjected to HPLC as in A–C. —,  $^3\text{H}$ -radioactivity; - - - - ,  $^{14}\text{C}$ -radioactivity; stippled area, SS-14-like immunoreactivity; . . . , acetonitrile gradient. The arrows indicate the elution position of synthetic (and anglerfish) SS-14 and [ $\text{Tyr}^7$  (Y, Gly $^{10}$  (G)) SS-14 ([Y $^7$ , G $^{10}$ ] SS-14) under the conditions employed.

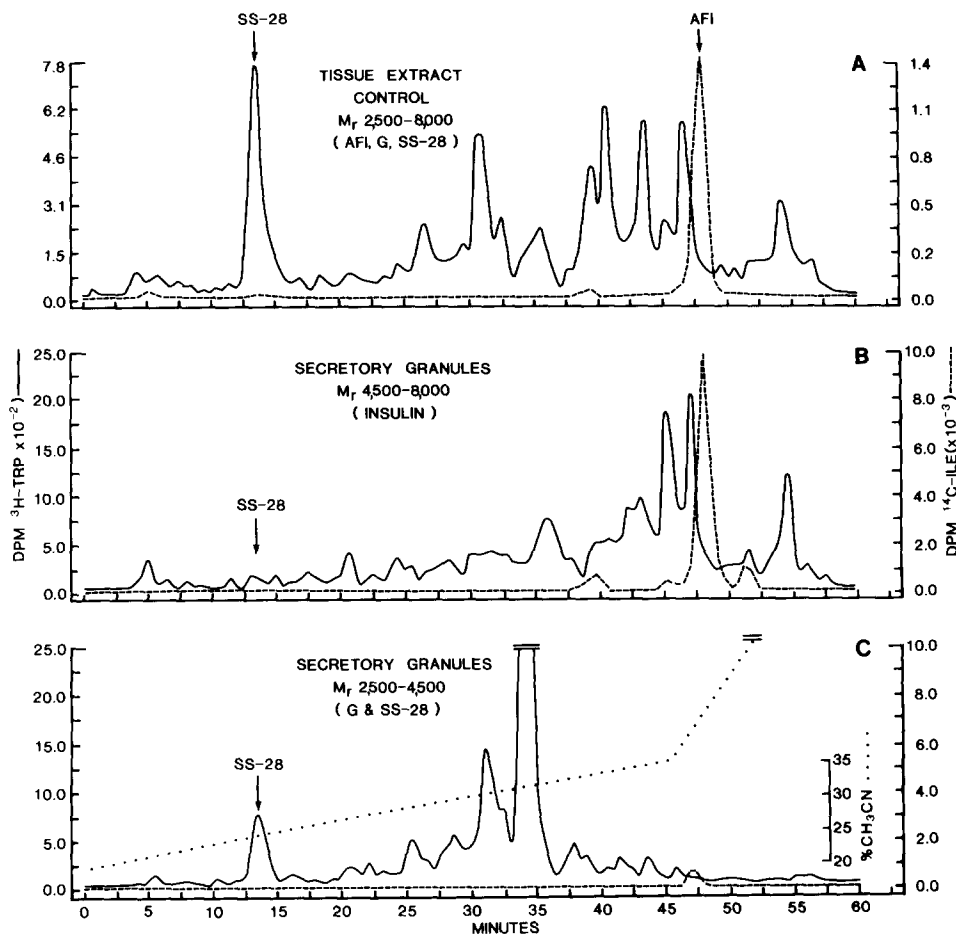


FIGURE 3 Action of complete granule lysate on proinsulin, proglucagon(s), and PSS-II. Preparation of secretory granules and incubation with prohormones was as described in Fig. 2. (A) Distribution of  $^3\text{H}$ - (—) and  $^{14}\text{C}$ - (----) radioactivity in the eluate after HPLC of a small portion of the combined peptides of  $M_r = 2,500-8,000$  from several 5-h pulse incubations of islets in the presence of [ $^3\text{H}$ ]tryptophan and [ $^{14}\text{C}$ ]isoleucine. (B)  $^3\text{H}$ - and  $^{14}\text{C}$ -radioactivity in the eluate after HPLC of peptides of  $M_r = 4,500-8,000$  generated by incubation of labeled prohormones with secretory granule lysate. (C)  $^3\text{H}$ - and  $^{14}\text{C}$ -radioactivity in the eluate after HPLC of peptides of  $M_r = 2,500-4,500$  generated from the same incubation as in B. All runs were performed using identical elution conditions. Fractions of 0.4 ml (0.5 min) were collected for assay of radioactivity. ····, acetonitrile gradient. Arrows indicate the elution positions of anglerfish SS-28 and insulin (AFI).

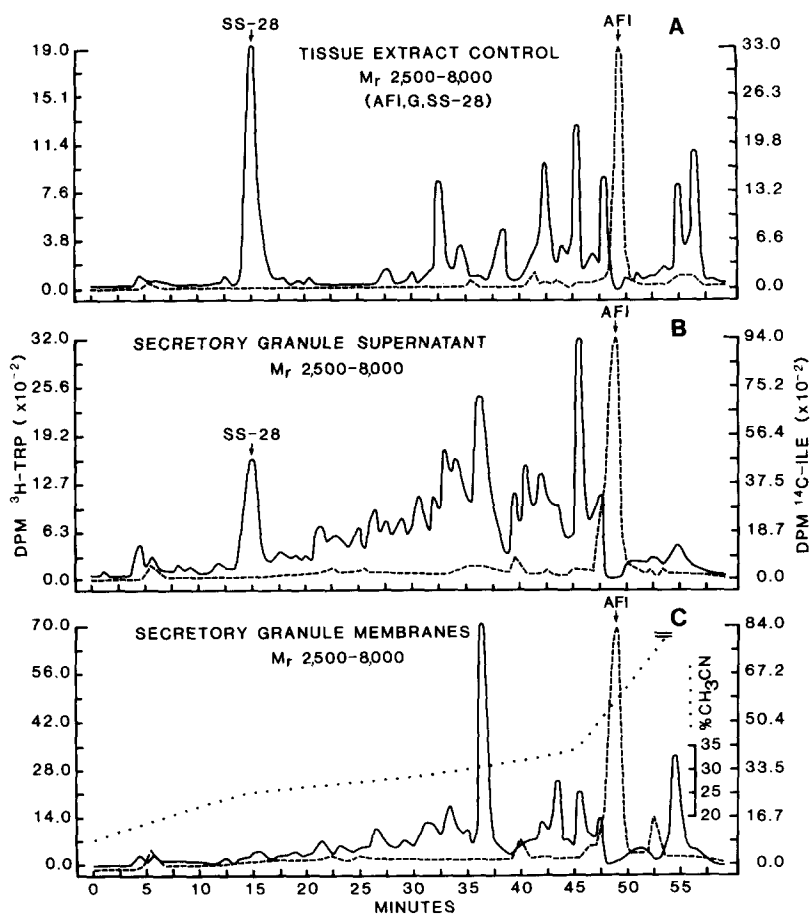


FIGURE 4 Action of granule lysate membranous and soluble components on proinsulin, proglucagon(s), and PSS-II. Preparation and incubation of granule membranes and soluble components were as described in Fig. 2. (A) Distribution of  $^3\text{H}$ - (—) and  $^{14}\text{C}$ - (----) radioactivity in the eluate after HPLC of the peptides of  $M_r = 2,500-8,000$  from several tissue extracts as in Fig. 3A. B and C, the distribution of  $^3\text{H}$ - and  $^{14}\text{C}$ -radioactivity in the eluate after HPLC of peptides of  $M_r = 2,500-8,000$  generated by incubation of radiolabeled prohormones of  $M_r = 8,000-15,000$  with granule supernatant (B) and membranes (C). Conditions for HPLC were the same in all runs. ····, acetonitrile gradient. Markers for the elution positions of anglerfish SS-28 and insulin are as in previous figures.

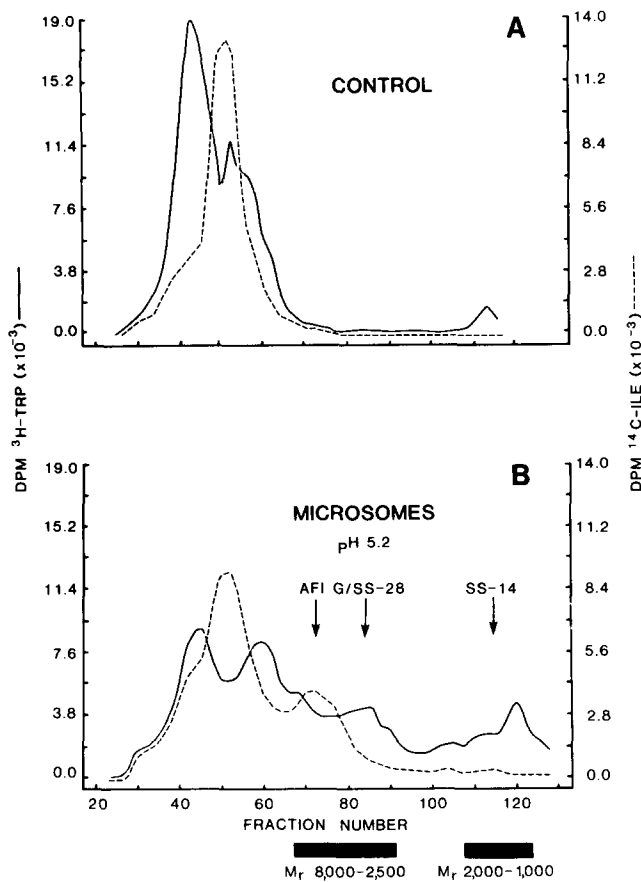


FIGURE 5 Islet prohormone conversion mediated by lysed islet microsomes. [ $^3\text{H}$ ]tryptophan- and [ $^{14}\text{C}$ ]isoleucine-labeled prohormones of  $M_r = 8,000\text{--}15,000$  were incubated 6 h in the absence or presence of lysed islet microsomes prepared from 58 mg tissue. Microsomal lysate protein to substrate protein ratio was 1:1. The resulting products were subjected to gel filtration and alternate 1.5-ml fractions were assayed for radioactivity. The distribution of  $^3\text{H}$ - (—) and  $^{14}\text{C}$ - (----) radioactivity in column eluates derived from running extracts of prohormones incubated in the absence (A) and presence (B) of microsomal lysate is shown. Arrows indicate the elution positions of anglerfish insulin (AFI), glucagon and SS-28 (G/SS-28), and SS-14. The bars below B indicate regions of the eluate recovered for HPLC analyses.

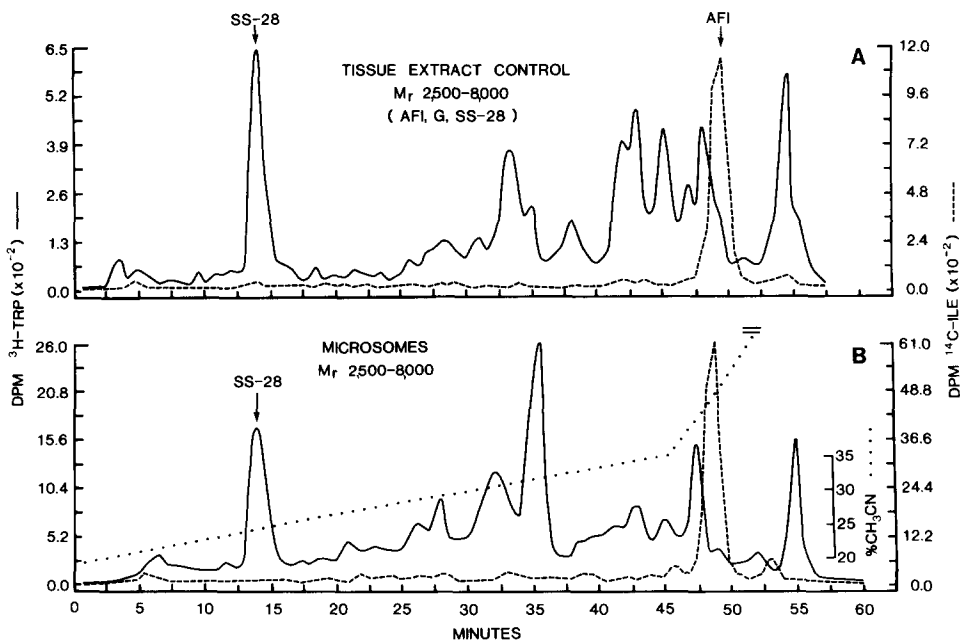


FIGURE 6 Action of complete microsomal lysate on proinsulin, proglucagon(s), and PSS-II. (A) HPLC of tissue extract peptides of  $M_r = 2,500\text{--}8,000$  as in Figs. 3A and 4A. (B) Distribution of  $^3\text{H}$ - (—) and  $^{14}\text{C}$ - (----) radioactivity in the eluate after HPLC of peptides of  $M_r = 2,500\text{--}8,000$  (bar, Fig. 5B) generated by incubation of labeled prohormones with lysed microsomes prepared from 50 mg of tissue. Identical elution conditions were used in both runs. ...., acetonitrile gradient; other markers are as in previous figures.

very similar to activity characterized previously in secretory granule lysates (1, 2).

Differences between granule- and microsomal-mediated conversion were observed, however, when cleavage products were analyzed by HPLC. The peptides of  $M_r 1,000\text{--}2,000$  and  $2,500\text{--}8,000$  produced by *in vitro* incubation of microsomal components with labeled prohormones were recovered from the appropriate regions of gel filtration eluates (bars, Fig. 5B). It should be noted that the major  $^3\text{H}$ -labeled peptide(s) which elutes in the  $M_r 1,000\text{--}2,000$  portion of the gel filtration eluate does not coelute with the SS-14 marker (Fig. 5B). When this peptide pool was subjected to HPLC, it was found that no  $^3\text{H}$ -labeled peptide having the RT of SS-14 was present (Fig. 2D). In contrast to secretory granule lysates, microsomal lysates were incapable of mediating the correct processing of PSS-I. However, when the peptides of  $M_r 2,500\text{--}8,000$  generated by incubation with microsomal components were examined by HPLC, several conversion products were readily identified (Fig. 6). It is evident that AF SS-28, AF insulin, and at least two glucagon-related products (RT 43.0 and 45.0) were generated by microsomal lysates. When the converting activities of microsomal membranes and soluble components were assayed separately, it was found that most of the proglucagon-to-glucagon and PSS-II-to-SS-28 converting activity was present in the soluble fraction (Fig. 7). However, the proinsulin-to-insulin converting activity of the soluble fraction was only slightly greater than that of the membranous fraction.

#### Relative Contribution of Golgi Complex and RER to Microsomal Converting Activity

To ascertain whether there was a difference in the relative contribution of Golgi components and elements of the endoplasmic reticulum to the overall converting activity of microsomes, Golgi complex and RER-enriched subfractions of microsomes were prepared and tested for the ability to convert islet prohormones. The subfractions generated were characterized by assay for thiamine pyrophosphatase (Golgi marker) (21) and electron microscopic examination. The data in Table I demonstrate that the uppermost bands recovered from the sucrose gradient (subfractions 1 and 2) had the

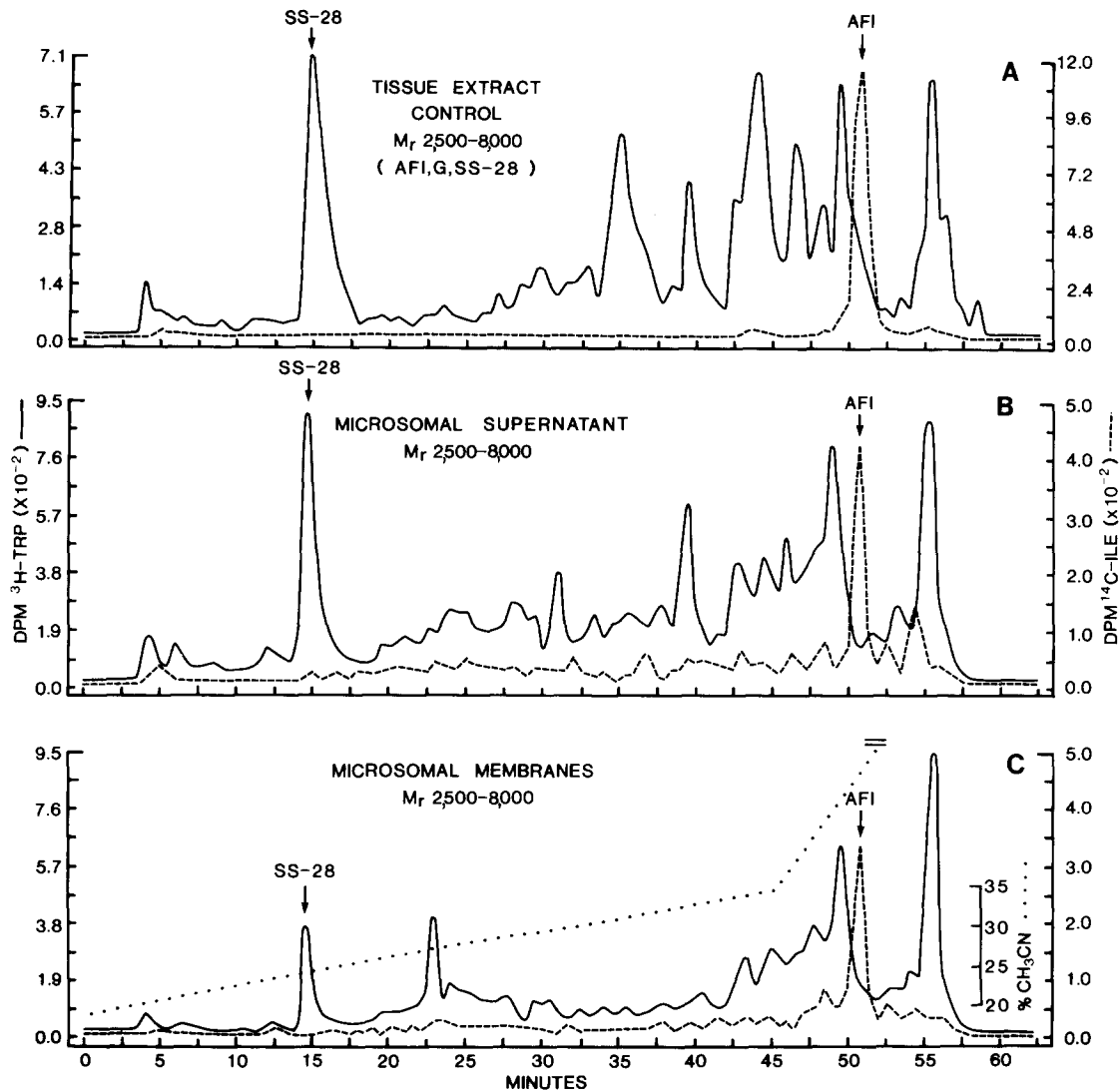


FIGURE 7 Comparison of the actions of soluble and membranous components of microsomes on proinsulin, proglucagon(s), and PSS-II. (A) HPLC of tissue extract peptides of  $M_r = 2,500-8,000$  as in Figs. 3A, 4A, and 6A. Microsomes prepared from 121 mg of tissue were lysed and separated into membranous and soluble components by centrifugation. Each component was incubated separately for 6 h with prohormones labeled with [ $^3\text{H}$ ]tryptophan and [ $^{14}\text{C}$ ]isoleucine. (B and C) Distribution of  $^3\text{H}$ - (—) and  $^{14}\text{C}$ - (---) radioactivity in the eluates after HPLC of the peptides of  $M_r = 2,500-8,000$  generated by incubations with soluble components (B) and membranes (C) from microsomes. . . . , acetonitrile gradient; other markers are as in previous figures.

highest Golgi membrane content, and that RER predominates in subfractions 4 and 5. Conversion assays were performed using subfractions 1-4 only; insufficient material was recovered from subfraction 5. The data in Table II document the results from these conversion assays. Data for SS-14 production are not included as microsome fractions were found to be incapable of accurately cleaving PSS-I to yield SS-14 (Figs. 5 and 2D). The results do demonstrate, however, that the microsomal subfractions were capable of mediating the generation of cleavage products having the labeling and molecular size characteristics of insulin, glucagon-related peptides, and SS-28. To determine whether the peptides generated by incubation of prohormones with microsomal subfractions could be identified as prohormone cleavage products, the peptides of  $M_r$  2,500-8,000 produced by all four subfractions in a single experiment were pooled for HPLC analysis. The HPLC results demonstrated that SS-28, insulin, and several of the glucagon-related products were present in this pool of products (data not shown). Given that the converting activity of the microsomal subfractions generates accurately cleaved

products, it is noteworthy that all subfractions produced products, and that there was no significant difference in the amount of products generated by any one of the subfractions as compared to the others (Table II).

## DISCUSSION

The results from experiments using complete secretory granule lysates demonstrate that these lysates mediate the accurate conversion of PSS-I to SS-14, PSS-II to SS-28, and proinsulin to insulin (Figs. 1-3). The observation that nearly all of the peptides generated during these conversion assays could be identified as accurate cleavage products suggests that nonspecific proteolysis is negligible in the assay system. Several glucagon-related peptides that are consistently found in tissue extracts were also generated by converting activity in secretory granule lysates (Figs. 1 and 3). These data confirm and extend our previous results (1, 2). In our earlier studies on the processing of PSS-I, conversion products generated by granule lysates were identified by gel electrophoresis (1). Using re-

TABLE I  
Analyses of Microsomal Subfractions

Sub-fraction	Thiamine pyrophosphatase	Electron microscopic analysis	
	Total recovered %	Smooth membranes $\mu\text{m}$	Rough membranes
1	31.6 ± 4.4 (4)	4,119.4	843.8
2	27.5 ± 4.0 (4)	4,402.4	1,358.6
3	19.2 ± 0.8 (4)	2,198.3	1,484.7
4	13.1 ± 0.8 (4)	1,560.0	3,739.5
5	8.6 ± 1.7 (4)	696.8	2,458.7

Microsomal subfractions were prepared, concentrated, and resuspended as described in Materials and Methods. Aliquots were removed for the thiamine pyrophosphatase assay, and portions of the remainder were fixed for electron microscopic analysis. Thiamine pyrophosphatase results are expressed as percent of total activity (micromoles of  $\text{PO}_4$ /milligram subfraction protein) recovered from each set of subfractions (mean ± standard error for the number of determinations in parentheses). For the electron microscopic analysis, six representative micrographs of the contents of each fraction were analyzed for the presence of smooth and rough membranes. The perimeters (in micromoles) of all vesicles that could be clearly identified as being either smooth or ribosome-studded were traced on a graphics-digitizing pad linked to a minicomputer. Data are expressed as the sum of all perimeters in each class.

TABLE II  
Comparison of the Prohormone-converting Activity of Microsomal Subfractions

Subfraction	Product	
	Insulin %	Glucagon/SS-28 %
1 (Golgi complex rich)	16.1 ± 1.3 (4)	11.4 ± 2.7 (4)
2	16.5 ± 2.5 (4)	10.5 ± 1.6 (4)
3	22.8 ± 1.7 (4)	13.6 ± 2.5 (4)
4 (RER rich)	22.0 ± 4.0 (4)	13.7 ± 2.6 (4)

Microsomal subfractions were recovered from discontinuous sucrose gradients, then concentrated, suspended, and lysed as described in Materials and Methods. Lysates were incubated 6 h at 30°C with labeled prohormones at a microsome protein to prohormone ratio of 2:1. Extracts were subjected to gel filtration and percent product was determined as previously described (2, 3). Data represent mean (± standard error) for the number of determinations shown in parentheses. Analyses of these data with both *t*-test and ANOVA methods revealed no significant differences between the converting activity of the subfractions.

verse-phase HPLC, a more resolute technique, we have now demonstrated that the major [ $^3\text{H}$ ]tryptophan-labeled product with a molecular size between 1,000 and 2,000 has a RT identical to that of synthetic SS-14 (Fig. 2). It was of interest to find that a second  $^3\text{H}$ -labeled peptide having the RT of synthetic [ $\text{Tyr}^7$ ,  $\text{Gly}^{10}$ ] SS-14 was also generated (Fig. 2, A-C). The generation of this peptide is considered to be an artifact of the *in vitro* conversion system because [ $\text{Tyr}^7$ ,  $\text{Gly}^{10}$ ] SS-14 cannot be identified in extracts of AF islet tissue (13, 14). As secretory granules from all islet cell types were present in these assays, the PSS-I to SS-14 converting enzyme would have access to PSS-II, and could cleave [ $\text{Tyr}^7$ ,  $\text{Gly}^{10}$ ] SS-14 from PSS-II or AF SS-28. If PSS-I and PSS-II are produced in different cell types, as discussed later, then it would be unlikely for this cleavage to occur either *in vivo*, or in intact tissue incubated *in vitro*.

Additional information regarding granule-mediated conversion of proglucagons was also obtained. It is evident that more glucagon-related peptides were observed after incubation of prohormones with secretory granule lysates (Figs. 3 and 4) than in a previous study (2). This can be attributed to

longer incubation times (6 h in the present study vs. 4 h previously), and the use of HPLC columns that are more highly resolute and provide much better recoveries of larger peptides. Further analysis of the proglucagon-converting activities of islet microsomes and secretory granules must await identification and characterization of the glucagon-related peptides generated in intact tissue and during *in vitro* conversion assays.

The primary purpose of the present study was to perform a thorough examination of prohormone converting activity in islet microsomes and microsomal subfractions and to compare microsomal converting activity with that in secretory granules. Results from previous studies indicated that, during *in vitro* incubations, islet microsomes have the ability to deplete labeled prohormones and generate products having molecular size and labeling characteristics of islet hormones (1, 2, 22). However, this microsomal converting activity had never been thoroughly characterized. As indicated in Results, the converting enzymes in microsomes are similar to those in granules with regard to pH optimum and susceptibility to inhibition by a cysteine proteinase inhibitor. When the products of conversion mediated by microsomal lysates were analyzed by HPLC and compared to products derived from granule mediated conversion, both similarities and differences were observed.

Microsomal lysates exhibited converting activity for PSS-II and proinsulin (Figs. 6 and 7), but, unlike secretory granule lysates (Fig. 2, A-C), were incapable of cleaving significant amounts of SS-14 from PSS-I (Fig. 2D). A significant proportion of the proinsulin and PSS-II converting activity in microsomal lysates was associated with the membranous component (Fig. 7C). All of the monitored prohormone-converting activities, except that for PS-II to SS-28 conversion, appear to be membrane-associated to some extent in both microsomes and secretory granules (Figs. 2C, 4C, and 7C). The converting activity of PSS-II was found to have no apparent membrane association in secretory granules (Fig. 4C). No attempt to compare relative specific converting activities in the membrane-associated vs. soluble fractions of either microsomes or secretory granules was made. Such comparisons would be inaccurate because of differential dissociation of enzymes from membranes during freeze-thaw lysis.

Membrane association of prohormone-converting enzyme(s) has also been demonstrated in secretory granules from rat anterior pituitary (10) and neurointermediate lobe (23) as well as in rat hypothalamic synaptosomes (24). Although the significance of membrane association of converting enzymes has not been investigated thoroughly, there is some evidence that newly synthesized prohormones may be granule membrane-associated as well (25, 26). We have recently obtained data which indicates that newly synthesized islet prohormones are microsome and secretory granule membrane-associated. If this proves to be a general phenomenon, then it would seem reasonable to propose that association of converting enzymes with RER/Golgi complex and secretory granule membranes may facilitate maintenance of juxtaposition between enzyme and substrate.

Analysis of converting activity in Golgi complex- and RER-enriched subfractions of microsomes demonstrated that accurate cleavage products were produced by the subfractions, and that there was no significant difference in converting activity between the various subfractions (Table II). It is thus evident that both RER and Golgi elements contain converting enzymes. These results are consistent with the hypothesis



proposed previously (2) that the converting enzymes may be synthesized at the RER along with the prohormones, then transferred into the RER cisternae, and transported along with the newly synthesized hormone precursors to the Golgi apparatus and secretory granules. However, the demonstration of converting activity in the RER and Golgi components does not necessarily imply that a significant proportion of overall converting activity occurs in these organelles in vivo. The in vitro assays in which conversion was observed were performed at pH 5.2. If the intracisternal pH of the RER and/or Golgi complex in the intact cells is near pH 7 or above, then it is unlikely that the converting enzymes would be very active in vivo. Moreover, it is possible that converting enzyme(s) destined to mediate processing in secretory granules may be less active when present in RER and Golgi complex due to conformational or structural constraints that impede expression of full activity. This could be exemplified by the converting enzyme(s) of PSS-I in AF islet, which was found to be active only in secretory granules. Because in vitro measurements of microsomal converting activity may not accurately reflect the situation in the intact cell, no attempt to compare relative specific converting activities in microsomes and secretory granules was made.

The data comparing the types and distribution of microsome and secretory granule converting activities does, however, provide new information regarding the mechanism of differential processing of PSS-I and PSS-II. When the results from both secretory granule- and microsome-mediated conversion of these precursors are considered, the clear implication is that (at least) two separate enzymes which have differential distribution or activity are involved. The converting enzyme of PSS-I is active and membrane-associated in secretory granules. The fact that this processing activity was not observed in microsomes implies that the converting enzyme for PSS-I is either not present or is inactive in RER and Golgi. If all converting enzymes are synthesized along with hormone precursors at the RER as proposed above, then it is probable that the PSS-I-processing enzyme is present, but inactive, in microsomes. The PSS-II-converting enzyme was active in both microsomes and secretory granules but was found to be membrane-associated only in microsomes. Other observations are also consistent with the proposal that two different enzymes may be involved in processing PSS-I and PSS-II. The PSS-I cleavage site is on the carboxyl side of an Arg-Lys sequence at positions -1 and -2 in PSS-I (27, 28), whereas the PSS-II cleavage site is on the carboxyl side of a single Arg at position -15 in PSS-II (27; manuscript in preparation). Moreover, preliminary results using immunohistochemical methodology indicate that PSS-I and PSS-II are synthesized in separate cells (Noe, B. D., and G. E. Bauer, unpublished data).

The differential subcellular distribution of converting activity of PSS-I and PSS-II would allow, under appropriate conditions, conversion of PSS-II to begin early during the transport process (i.e., in the rough endoplasmic reticulum and/or Golgi complex) whereas the predominant PSS-I processing would occur distally in the secretory granules. A somewhat analogous situation has been described in the rat pituitary intermediate lobe by Glembotski (29), who demonstrated that processing of pro-opiomelanocortin to release  $\beta$ -lipotropin occurred in the microsomes, whereas most of the conversion of  $\beta$ -lipotropin into  $\beta$ -endorphin-sized peptides occurred in secretory granules. Finally, the results of the present study may be relevant to the differential processing of prosomatostatin which occurs in mammals. Results from numerous studies have demonstrated differential distribution of both SS-28 and SS-14 in various tissues (30-33), and release of both peptides into plasma (33-36). It is probable that the amount of either peptide that is produced in various tissues depends on differential cleavage of prosomatostatin. To determine whether the mechanism in mammals involves two separate enzymes, as is apparent in fish islets, will require additional investigation in mammalian systems.

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