Studies on the Conversion of Proinsulin to Insulin

III. STUDIES IN VITRO WITH A CRUDE SECRETION GRANULE FRACTION ISOLATED FROM RAT ISLETS OF LANGERHANS*

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SUMMARY

Proinsulin is converted to insulin in an impure secretion granule fraction prepared from rat islets of Langerhans that have been labeled before homogenization with [3H]leucine or [3H]arginine. During incubation of this particulate fraction at pH 6.3 and 37°, the initial rate of conversion of the endogenous labeled proinsulin is similar to that observed in whole islets, while externally added labeled proinsulin is not cleaved. Only intact granules catalyze conversion, and thus the pH optimum of about 6.0 for this process corresponds closely to that for granule stability. The most rapid in vitro cleavage of endogenous proinsulin is observed when the islets have been prelabeled with [3H]leucine for 30 min, followed by a 15-min "chase" to allow time for the transport of newly synthesized proinsulin to the Golgi apparatus and new secretory granules. Several proteinase inhibitors, including soybean trypsin inhibitor, pancreatic trypsin inhibitor, diisopropyl fluorophosphate, N-α-p-tosyl-L-lysine chloromethyl ketone·HCl, benzamidine, β-nitrophenyl-β'-guanidinobenzilate·HCl, N-ethylmaleimide, and iodoacetate, do not inhibit conversion in vitro, possibly due to a lack of permeability of the granules to some of these substances; high concentrations of p-chloromercuribenzoate completely inhibit conversion.

The products of in vitro conversion have been characterized by polyacrylamide gel and thin layer electrophoresis as rat insulins I and II and their corresponding C-peptides. The residual proinsulin fraction after incubation consists mainly of partly cleaved intermediate forms. The islets are prelabeled with [3H]arginine before preparation of the granule fraction, in vitro conversion is accompanied by the release from the cleavage regions of free arginine rather than dipeptides of arginine or lysylarginine.

Granule preparations disrupted by repeated freeze-thawing lose their ability to introduce cleavages in intact proinsulin but are still able to rapidly remove COOH-terminal arginine residues from lightly trypsinized proinsulin. A low level of trypsin-like activity, as indicated by the slow cleavage of N-α-tosyl-L-[methyl-3H]arginine methyl ester, can still be detected in the disrupted preparations. These results are in accord with the hypothesis that enzyme(s) having trypsin-like and carboxypeptidase B-like activity exist in the secretory granules of the β-cells and participate in the conversion of proinsulin to insulin. The nature and precise subcellular localization of these proteolytic enzymes, as well as their possible relationship to the exocrine pancreatic proteases, remain to be clarified.

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MATERIALS AND METHODS

Islets of Langerhans were isolated by collagenase digestion of the pancreas of two normal rats (Sprague-Dawley, female, 200 to 300 g) as described elsewhere (11). L-[4,5-3H]leucine (22 Ci per mmole) and L-[5-3H]arginine monohydrochloride (8.7 Ci per mmole) were obtained from the Radiological Centre, Amersham, England. The N-α-tosyl-l-lysyl-l-histidine methyl ester (280 mCi per mmole) was obtained from Cal Atomics Corporation, Los Angeles, Calif. Unless stated otherwise proinsulin and insulin were separated by gel filtration on columns (1 × 50 cm) of Bio-Gel P-30 (Bio-Rad Laboratories, Richmond, Calif.), equilibrated with 3 M acetic acid (1.0 to 1.5 ml fractions were collected). The radioactivity of fractions was determined by counting aliquots in toluene-Triton scintillation fluid (12) in a Packard Tri-Carb liquid scintillation spectrometer. Thin layer chromatography was carried out on sheets of Eastman No. 6064 cellulose using concentrated NH₄OH-propanol (2:3) as the solvent (8).

Incubation and Labeling of Islets—Incubation was carried out in small plastic culture dishes. Freshly isolated rat islets were transferred to 50 or 100 μl of modified Hank’s medium, pH 7.4, containing essential amino acids (12), and were preincubated for 10 min at 37°C. [3H]Leucine (5 to 20 μCi) was then added to the small incubate and thoroughly mixed by blowing obliquely against the fluid drop from a micropipette. The islets usually were incubated with [3H]leucine for 30 min, washed several times with medium containing unlabeled leucine, and then incubated 15 min at 37°C in this medium to allow for the newly synthesized proinsulin into Golgi structures and secretion granules.

Preparation of Crude Secretion Granule Fraction—Prelabeled or unlabeled islets were homogenized manually in a Dounce homogenizer, size 20, with Teflon pestle (Kontes Ltd., Vineland, N. J.) in 0.3 ml of buffer containing 0.25 M sucrose, 1 M Ficoll (Pharmacia, Uppsala, Sweden), 2% bovine serum albumin, and 0.01 M potassium glycerophosphate, pH 6.0. The homogenate was centrifuged at 800 × g for 10 min to sediment nuclei, debris, and intact cells. The sediment was rehomogenized in 0.2 ml of the same buffer and centrifuged. The combined supernatants were then transferred by layering to a cellulose nitrate ultracentrifuge tube (15 × 15 inches) containing a lower layer consisting of 0.1 ml of 2.0 M sucrose, 5% Ficoll, 2% bovine serum albumin, 0.01 M potassium glycerophosphate, pH 6.0, and an intermediate layer of 0.15 ml of 0.6 M sucrose, 1% Ficoll, 2% bovine serum albumin, and 0.01 M potassium glycerophosphate, pH 6.0. After centrifugation at 5 × 10⁶ g min a visible layer of particles could be seen on the top of the 2.0 M sucrose layer. This zone was transferred to a small tapered centrifuge tube coated with silicone (Siliclad, Clay Adams, Parsippany, N. J.) containing 0.5 ml of the following buffer: 160 mM KCl, 5 mM NaCl, 5 mM MgCl₂, 4 mM cysteine, 2% cysteine, 2% bovine serum albumin, 0.01 M potassium glycerophosphate, pH 6.3. This suspension was incubated at 37°C for 1 to 5 hours to observe the transformation of proinsulin to insulin (7). After incubation, 0.5 ml of the crude granule fraction was added and sufficient glacial acetic acid to give a final concentration of 3 M. Any precipitate was centrifuged off and dissolved. The fractions were collected in silicone-treated or albumin-coated tubes to minimize adsorption of the small amounts of radioactive proteins to the glass surfaces.

For inhibition studies the following substances were used: diisopropyl fluorophosphate (1 mM, 2.5 mM, 10 mM); N-ethylmaleimide (10 mM); benzamidine (10 mM); N-ethylmaleimide (10 mM); iodoacetamide (10 mM); and the trypsin-treated material was rechromatographed on a Bio-Gel P-30 column. The fractions containing insulin and C-peptide were collected. The inhibitors were done at pH 6.3, except in the case of p-chloromercuribenzoate which is not soluble at a concentration of 1 mM at pH 6.3. Incubation with this substance was done at pH 7.0 with a control incubated at this pH.

Separation of C-peptides from Insulin—Since insulin and C-peptides have nearly the same elution volumes on gel chromatography (13) these substances were separated and quantitated by paper electrophoresis on Whatman No. 1 paper in 30% formic acid at 5 volts per cm for 5 hours, followed by scanning of the dried strips in a windowless chromatogram strip-scanner (the Technical Measurement Corp.). In some experiments electrophoresis was carried out instead on cellulose plates (20 × 20 cm) (Eastman No. 6064, Eastman Kodak Co., Rochester, N. Y.) in 30% acetic acid for determination of radioactivity.

Polyacrylamide gel electrophoresis was carried out at pH 8.5 according to Ornstein and Davies (14, 15). To extract the proteins after electrophoresis the gels were cut into 1-mm sections with a small device similar to a wire egg slicer. Two slices were placed in each glass scintillation vial. The slices were dried by heating the unscanned bottles for 1 hour at 60°C in an oven. To each bottle 0.7 ml of 88% formic acid was added. The bottle was tightly capped and heated overnight in an oven (at 60°C). The bottles were cooled and 15 ml of Bray’s scintillator solution (16) were added. After standing for at least 20 min, the bottles were shaken and counted. The recovery of the method was 80 to 90%.

N-α-Tosyl-l-arginine Methyl Ester Assay (17)—Before use in the assay it was necessary to extract the labeled N-α-tosyl-l-arginine methyl ester with toluene to reduce background extractable radioactivity. In typical assays 10 μl of buffer (0.1 M Tris-HCl, pH 7.5), 10 μl of enzyme preparation in Tris-HCl buffer, and 10 μl of N-α-tosyl-l-[3H]arginine methyl ester solution (0.1 mM) in water were mixed in a glass scintillation vial and the assay was performed as described by Roffman et al. (17). The results were corrected for spontaneous hydrolysis of the substrate by means of appropriate blank assays carried out under similar conditions.

Detection of Carboxypeptidase B Activity in Crude Granule Fractions—About 1000 islets from six rats were incubated with labeled arginine for 8 hours. They were homogenized in acid-ethanol and extracted as above. After gel filtration of the extract, fractions of the proinsulin peak were combined and evaporated to dryness in a flash evaporator and the residue was redissolved in 0.2 ml of 0.1 M Tris-HCl, pH 8.0. 1.0 M tosylamide 2-phenylethyl chloromethyl ketone-treated trypsin (1 μg) was then added and the mixture was incubated for 30 min at 37°C (2, 9, 10). After incubation, 0.5 ml of 3 M acetic acid was added and the trypsin-treated material was redchromatographed on a Bio-Gel P-30 column. The fractions containing insulin and C-peptide were collected. The inhibitors were done at pH 6.3, except in the case of p-chloromercuribenzoate which is not soluble at a concentration of 1 mM at pH 6.3. Incubation with this substance was done at pH 7.0 with a control incubated at this pH.
peptide were combined and evaporated to dryness. The residue was redissolved in 0.2 ml of Tris-HCl, pH 8.0, and used as a substrate for measurement of carboxypeptidase B-like activity.

A crude granule fraction was prepared from about 500 islets, without prior labeling, as described above. The pellet from the 5 x 10^4 g min centrifugation was resuspended in 0.2 ml of 0.1 M Tris-HCl, pH 7.0, dispersed by gentle mechanical homogenization, and then rapidly frozen and thawed six times. Aliquots of this homogenate and of the substrate described above were combined and incubated at 37°C. At various times the reaction was stopped by addition of 0.5 ml of 3 M acetic acid. Unlabeled L-arginine (200 μg) and carrier insulin (0.5 mg) were added. The samples were subjected to gel chromatography on Bio-Gel P-30 as described above without prior extraction. Pancreatic carboxypeptidase B (diisopropyl fluorophosphate-treated, Worthington Biochemical Corp., Freehold, N. J.) was added as a control to one aliquot of the substrate at a concentration of 20 μg per ml. In this case incubation was carried out for 20 min. The crude granule fraction of the human islet cell adrena was prepared and treated in the same way as the rat granule fraction.

**RESULTS**

**Attempts to Isolate Secretion Granule Fractions**—Several methods were used to attempt to separate clean secretion granules from islet homogenates. In initial experiments whole islet homogenates were fractionated on continuous sucrose gradients (18). Although these preparations were not examined microscopically, several observations indicated that the fractions of the gradient containing secretion granules were heavily contaminated with microsomes. A comparison of the distribution of radioactivity after preincubation of islets with [3H]leucine for 10 min or for 3 hours is shown in Fig. 1. In both cases the sedimentable activity was distributed similarly at a density of about 1.20, suggesting that the vesicles derived from the rough and smooth endoplasmic reticulum, which contained only labeled proinsulin after a short pulse (see Fig. 1 top, inset), sediment along with secretion granules which probably contain most of the sedimentable insulin-related radioactive proteins after the longer labeling period (18, 19).

Several approaches were tried to improve the separation of the microsomes and secretion granules without success, including the addition of a prior step of differential centrifugation (20), filtration with standard Millipore filters, and application of procedures for dissociation of ribosomes from vesicles of rough endoplasmic reticulum by means of puromycin treatment (21). In vitro incubation under various conditions of prelabeled granule fractions prepared by these different methods did not result in measurable conversion of proinsulin to insulin and C-peptide.

**Conversion Studies with Crude Secretion Granule Fraction Containing [3H]Leucine-labeled Proinsulin**—The following conversion studies were carried out with the crude secretion granule fraction prepared as described under "Materials and Methods." When [3H]leucine-prelabeled islets were homogenized gently (using manually operated devices) a crude secretion granule fraction which retained the ability to convert proinsulin to insulin in vitro (Fig. 2) could be separated by centrifugation onto a sucrose cushion. In several experiments about 38% of the endogenously labeled proinsulin contained in the granule fraction was converted to material having the gel exclusion characteristics of rat insulin in 1 hour (Table I). When the labeling period of the islets prior to preparation of the granule fraction was reduced to only 7 min without any "chase" incubation, the labeled protein in the crude secretion granule fraction consisted only of proinsulin, which presumably was localized mainly in vesicles derived from the rough endoplasmic reticulum. When these preparations were incubated in vitro essentially no conversion of proinsulin to insulin was observed (Table I). Prelabeling islets for relatively long intervals did not enhance the rate of conversion in vitro. Thus, for most experiments, the 30-min pulse-15-min chase procedure was used for prelabeling the islets.

The effects of several known protease inhibitors were tested in this in vitro conversion system. The protein protease inhibitors, such as pancreatic trypsin inhibitor and soybean trypsin inhibitor were not effective. The failure of these substances to inhibit the reaction may have been related to the probable impermeability of the granule membrane to such large peptides; for example, externally added 125I-labeled bovine proinsulin also evidently did not permeate the granules, as it was not converted in this system (7). Similarly, preincubation of the granule fraction for 1 hour at room temperature with N-ethylmaleimide, sodium iodoacetate, diisopropyl fluorophosphate, benzamide, NPBG, and TLCK in relatively high concentrations also failed to inhibit significantly the observed in vitro conversion. However, p-chloromercuribenzoate in relatively high concentrations (1 mM) completely inhibited conversion. This effect was not due simply to lysis of the granules as was determined by measuring the amount of sedimentable radioactive protein after incubation.
mide gel electrophoresis at pH 8.9 (Fig. 3), three major peaks were observed corresponding in position to standards of the two known rat insulins and with the tracker dye band which previously has been shown to contain the very acidic rat C-peptides (22). The rat insulins occurred in a ratio of 58% (I) to 42% (II), as was noted previously (22). Similar results were obtained on thin layer cellulose electrophoresis in 30% formic acid, although the two rat insulins were not fully separated. The proportion of total radioactivity found in the insulin and the C-peptide fractions agreed well between these two methods, the figures for percentage of C-peptide radioactivity being 43 and 45, respectively. According to the known distribution of leucine residues between the rat insulins (6 residues) and C-peptides (5 residues), this figure theoretically should be 45.4%. The close agreement of the observed values suggests that after its cleavage from proinsulin the C-peptide is retained within the granules.

In several earlier experiments (see for example Fig. 1, lower panel) the percentage of radioactivity in insulin and C-peptide was measured in both the sedimentable material and the soluble material remaining at the top of the gradient. In these cases, the C-peptide comprised from 88 to 69% of the combined insulin and C-peptide radioactivity in the soluble material and only about 34% in the sedimentable fraction. These nonstoichiometric relationships suggest that a variable degree of leakage of the C-peptide from the sedimentable material had occurred. This was presumed to be due to mechanical damage of the granules in the initial homogenization step. In later experiments carried out with a loose fitting glass-Teflon homogenizer, leakage of C-peptide was not observed. These results suggest that damage to the secretion granule membrane may result in greater losses of C-peptide than insulin, perhaps due to the fact that much of the insulin contained in the granules may be precipitated or aggregated into microcrystals (23) while the C-peptide probably remains in solution.

**Characterization of in Vitro Conversion Products**—In experiments similar to the one shown in Fig. 2, incubated granules were sedimented and extracted. After gel filtration of the extracts the proteins of the insulin peak were characterized by polyacrylamide gel and thin layer cellulose electrophoresis. On polyacrylamide gel electrophoresis at pH 8.9 (Fig. 3), three major peaks were observed corresponding in position to standards of the two known rat insulins and with the tracker dye band which previously has been shown to contain the very acidic rat C-peptides.

**Table I**  
Conversion of proinsulin to insulin and C-peptide during incubation of the crude granule fraction

<table>
<thead>
<tr>
<th>Labeling conditions</th>
<th>Incubation time in vitro</th>
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<tr>
<td></td>
<td>1 hr</td>
</tr>
<tr>
<td>A. 37°</td>
<td></td>
</tr>
<tr>
<td>Pulse 30 min; chase 15 min</td>
<td>37°</td>
</tr>
<tr>
<td>Pulse 7 min; no chase</td>
<td></td>
</tr>
<tr>
<td>Pulse 30 min; chase 60 min</td>
<td>29</td>
</tr>
<tr>
<td>B. 22°</td>
<td></td>
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<tr>
<td>Pulse 30 min; chase 15 min</td>
<td>15</td>
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* All values are expressed as the percentage of radioactivity of proinsulin initially present at zero time that appeared as insulin and C-peptide (combined) during the specified incubation period. Temperatures given under A and B refer to in vitro incubation conditions.

(22).
Fig. 4. Polyacrylamide gel electrophoresis (pH 8.9) of the radioactive proteins in the proinsulin peak obtained on gel filtration of a prelabeled crude granule fraction after incubation for 5 hours in vitro in an experiment similar to that shown in Fig. 2. The positions of the main constituents of rat proinsulin obtained from rat insulin crystals (22), as well as the two rat insulins, are shown below the graph. In this system intact rat proinsulins I and II migrate together in the band marked below the graph. In this system intact rat proinsulins I and II, rat insulin crystals (22), as well as the two rat insulins, are shown in the positions of the main constituents of rat proinsulin obtained from in vitro in an experiment similar to that shown in Fig. 2. The positions of the main constituents of rat proinsulin obtained from rat insulin crystals (22), as well as the two rat insulins, are shown below the graph. In this system intact rat proinsulins I and II migrate together in the band marked below the graph.

Fig. 6. Identification of [3H]arginine as the major constituent of the low molecular weight fraction released from [3H]arginine-labeled proinsulin during incubation of the crude granule fraction in vitro, as shown in Fig. 5. Unlabeled arginine and diarginine were added to the sample as carriers. Thin layer chromatography was carried out as described under “Materials and Methods.” After staining with ninhydrin, the indicated sections of the chromatogram were scraped from the plates, extracted with 50% acetic acid, and counted in a liquid scintillation counter.

Fig. 5. Conversion of [3H]arginine-labeled proinsulin (Fractions 17 to 23) to insulin-like components during a 4-hour incubation in vitro at pH 6.3 and 37° of a crude granule fraction prepared from rat islets prelabeled with [3H]arginine before homogenization. The products were separated by gel filtration on columns of Bio-Gel P-30 in 3 M acetic acid. The low molecular weight material appears in Fractions 35 to 45. (Approximately 400 islets were incubated for 30 min at 37° with [3H]arginine and then for an additional 15 min with unlabeled arginine. The islets were then homogenized and the crude granule fraction was prepared as described under “Materials and Methods.”)

have shown that progressive accumulation of intermediate forms in the proinsulin fraction occurs as the incubation time is extended (3, 24).

Studies with Crude Granule Fractions after Labeling with [3H]-Arginine—In order to gain further insight into the precise nature of the proteolytic events involved in the conversion of proinsulin to insulin we have prelabeled rat crude secretion granules with [3H]arginine. This amino acid occupies three of the four linkage positions on either side of the C-peptide in proinsulin, as well as occurring once in the B chain at position B22 and once in the C-peptide at position C68 (25, 26). Conversion to insulin results in the excision of the 4 basic linking residues, but it is not known whether these are released as dipeptides or single residues. Fig. 5 shows the Bio-Gel P-30 elution profiles obtained before and after incubation of a [3H]arginine-prelabeled crude granule fraction for 4 hours in vitro. As was the case with the leucine-labeled granule fractions, extensive conversion of proinsulin to insulin occurred. In this instance, however, approximately three-fifths of the radioactivity derived from proinsulin appeared in a low molecular weight peak (Fractions 35 to 45, Fig. 5). This material was collected and examined in a thin layer chromatographic system which permits the resolution of free arginine or lysine from their respective dipeptides (Fig. 6). No radioactivity was found at the position of diarginine or lysylarginine, suggesting that the arginine residues are released from proinsulin in a stepwise fashion as would be the case also with an exopeptidase such as carboxypeptidase B.

Studies with Lysed Crude Granule Fractions—The in vitro conversion reaction is strongly dependent upon the integrity of the particulate elements involved in various procedures which would be expected to disrupt these, such as treatment with ionic or non-ionic detergents, sonication and repeated freezing and thawing, or freeze-drying, markedly inhibited the conversion, as we reported previously (7). Likewise lowering or raising the pH away from 6.3 sharply decreases the extent of conversion observed in vitro, presumably due in part to lysis of the granules, the stability of which is known to be highly pH dependent (27, 28). Thus at pH 4 or 8 essentially all of the labeled protein was released into the supernatant fraction during incubation at 37°.

This nearly total loss of converting activity also was observed when exogenous substrates such as [125I]labeled bovine proinsulin or [3H]arginine-labeled rat proinsulin were added to lysed granules prepared either from [3H]leucine-prelabeled or unlabeled islets.
There was no evidence of nonspecific degradation of the proinsulin. However, using a sensitive microassay for trypsin-like enzymes based on the hydrolysis of N-α-tosyl-L-arginine methyl ester a very small amount of trypsinic activity could be detected in the lysed granule preparations.

Although trypsin-like hydrolysis of the natural substrate could not be demonstrated in the lysed granule preparations, a carboxypeptidase activity that removed COOH-terminal basic residues from lightly trypsin-treated [3H]arginine labeled proinsulin was demonstrable. Proinsulin labeled in this way contains [3H]arginine methyl ester (17) a very small amount of trypsinic activity could be detected in the lysed granule preparations.

Carboxypeptidase activity was detected in a lysed granule fraction prepared from a human islet cell adenoma that actively converted proinsulin to insulin in biosynthetic experiments. Sufficient material was available from the tumor to determine the pH optimum (pH 8.0). The activity of this enzyme was inhibited by diisopropylfluorophosphate and TLCK but not by N-ethylmaleimide, p-chloromercuribenzoate, iodoacetamide, soybean trypsin inhibitor, or pancreatic trypsin inhibitor. However, a concentrate of this activity prepared by ammonium sulfate fractionation failed to convert [3H]arginine labeled bovine proinsulin to insulin. The nature of this trypsinic-like activity and its relationship to the conversion mechanism thus remains to be clarified.

DISCUSSION

Although insulin-containing secretion granule fractions have been separated from islet tissues of both fish (29, 30) and mam-

Fig. 7. Bio-Gel P-30 gel chromatography of the reaction products from lightly trypsin-treated [3H]arginine-labeled rat proinsulin. The fractions containing insulin and C-peptide (tubes 24 to 29) were combined and the acetic acid removed in vacuo. The residue was dissolved in Tris buffer and used as a substrate in the experiment shown in Figs. 8 and 9.

Fig. 8. Bio-Gel P-30 chromatogram showing the release of free [3H]arginine by treatment with excess carboxypeptidase B of the [3H]arginine-labeled substrates prepared by mild trypsin treatment of [3H]arginine-labeled rat proinsulin as shown in Fig. 7. Fractions 35 to 39 were combined, dried, and the residue subjected to thin layer chromatography as in the experiment shown in Fig. 6. Only free [3H]arginine was found in the low molecular weight fraction.

Fig. 9. Release of free [3H]arginine during incubation with an islet granule lysate of the [3H]arginine-labeled substrates prepared by mild trypsin treatment of [3H]arginine-labeled rat proinsulin, as shown in Fig. 7. The amount of arginine released was determined by gel chromatography of aliquots removed at various times during the incubation and was expressed as the percentage of the radioactive arginine that was released by excess carboxypeptidase B. For comparison is shown the amount of arginine released during incubation of the same labeled substrate preparation with the supernatant fraction remaining after centrifugation of the islet postnuclear supernatant to sediment the granule fraction.

There was no indication of nonspecific degradation of the substrate during any of these incubations.
secretion from the cisternae of the rough endoplasmic reticulum is a process of biosynthesis and conversion of proinsulin to insulin. Electron microscopy and autoradiographic techniques have provided strong evidence that β cells are similar to many other secretory cells in their ability to convert endogenously labeled proinsulin. However, studies using specific inhibitors and radioactive labeling have shown that this conversion initiates 15 to 20 min after biosynthesis, during which time transfer to the Golgi region is inhibited by addition of antimycin A and other inhibitors of energy metabolism during the critical 30-min period immediately after biosynthesis, during which time transfer to the Golgi apparatus takes place. Moreover, the conversion of newly synthesized proinsulin is inhibited by addition of antimycin A and other inhibitors of energy metabolism, exhibiting a characteristic pseudo-first order time course with a half-time of about 1 hour. This rather slow rate of processing is consistent with the hypothesis that while biosynthesis of the proinsulin peptide chain, a time lag consistent with a conversion of 15 to 20 min after biosynthesis, during which time transfer to the Golgi region occurs. Furthermore, this conversion begins to occur 15 to 20 min after biosynthesis, during which time transfer to the Golgi region is inhibited by addition of antimycin A and other inhibitors of energy metabolism, exhibiting a characteristic pseudo-first order time course with a half-time of about 1 hour (1). This rather slow rate of processing is consistent with the hypothesis that while conversion may be initiated in the Golgi apparatus it continues within maturing secretion granules for several hours after these have formed within the Golgi tubules, thus ensuring the necessary specificity and sequestration of this process.

The evidence presented here and in a previous report (7) of the in vitro conversion of proinsulin to insulin in a crude secretion granule fraction lends further support to the above conclusions. In this system granules containing proinsulin, labeled with either tritiated leucine or arginine, convert the proinsulin to insulin at rates that are initially comparable to those observed in vivo. With time, however, the rate of conversion slows and eventually stops short of the complete conversion observed in pulse-chase experiments with intact cells. This may be due in part to suboptimal conditions of incubation as well as to altered permeability of the granule membranes leading to the development of unsuitable conditions within the particles for continued reaction. Some leakage of substrate or enzymes from the particles also may occur. Despite these limitations, this preparation is useful for studying the requirements of the process and has provided a means for further delineating the nature of the enzymes involved.

Structural studies of several mammalian proinsulins (9, 10, 37), as well as the available evidence on the structures of the two rat proinsulins and C-peptides (13, 25, 26), indicate that a pair of basic residues located at the ends of the interchain connecting polypeptide and linking this region to the A and B chains in proinsulin is excised during conversion, giving rise to insulin and the C-peptide. Cleavage at these sites clearly must be initiated by a trypsin-like endopeptidase cleaving mainly, if not exclusively, on the carboxyl side of the second basic amino acid of each pair (8). The removal of the basic residues can then be accomplished by an exopeptidase having properties similar to carboxypeptidase B (8). In the case of the mouse, it has been difficult to obtain highly purified preparations free of measurable contamination (32). Several fractionation methods were tried in the studies described here, including zonal and isopycnic density gradient centrifugation, Millipore filtration, and puromycin pretreatment, without successfully eliminating rough and smooth microsomes and lysosomes. The similar densities and sedimentation properties of all of these particles make it very difficult to separate. This technical limitation has hampered efforts to localize precisely the newly synthesized secretory products and to define the orderly sequence of movement of these materials within organelles in the β-cell during its secretory cycle. However, studies using electron microscopy and autoradiographic techniques have provided strong evidence that β cells are similar to many other secretory cells (33–35) in transferring the proteins destined for secretion from the cisternae of the rough endoplasmic reticulum to secretory granules via the Golgi apparatus (18, 19).

Previous studies from this laboratory on the time course of the biosynthesis and conversion of proinsulin to insulin have indicated that conversion begins to occur 15 to 20 min after biosynthesis of the proinsulin peptide chain, a time lag consistent with a requirement for transfer of the proinsulin to the Golgi region (1, 5). Moreover, the conversion of newly synthesized proinsulin is inhibited by addition of antimycin A and other inhibitors of energy metabolism during the critical 30-min period immediately after biosynthesis, during which time transfer to the Golgi apparatus takes place (5, 34, 36). Once proteolysis of proinsulin begins in intact cells, the reaction proceeds independently of energy metabolism, exhibiting a characteristic pseudo-first order time course with a half-time of about 1 hour (1). This rather slow rate of processing is consistent with the hypothesis that while conversion may be initiated in the Golgi apparatus it continues within maturing secretion granules for several hours after these have formed within the Golgi tubules, thus ensuring the necessary specificity and sequestration of this process.

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To prove the validity of the preceding hypothesis regarding the nature of the converting enzymes it is necessary to demonstrate the presence of both trypsin and carboxypeptidase B-like activities within the β-granules. We have had considerable difficulty demonstrating the presence of trypsin-like activity in secretion granule fractions that have been lysed by repeated freeze-thawing. Such preparations almost completely lose the ability to convert endogenously labeled proinsulin. Nonetheless, as shown here, these preparations contain low levels of N-α-tosyl-L-arginine methyl ester splitting activity consistent with the presence of trypsin activity in some form. Moreover, recent studies of Dr. R. E. Smith using electron microscopic histochemical localization techniques have demonstrated the deposition of an electron-dense trypsin reaction product within secretory granules in rat β-cells (40). These deposits are especially prominent in the granules surrounding the Golgi apparatus.

Our failure to detect proinsulin splitting (endopeptidase) activity in the lysed granule preparation may be due to trivial causes, i.e. enzyme instability, substrate dilution, etc. On the other hand, this may be a clue indicating that the endopeptidase activity is a component of the inner membrane surface of the secretion granule and that it may become sequestered from the substrate through resealing of the granule membrane during the freeze-thawing procedure. Likewise, the failure of all of the inhibitors tested, except p-chloromercuribenzoate, to affect conversion in either intact islets (24) or in the in vitro granule system may stem from a failure of these substances to penetrate the granule membranes.

On the other hand, the studies with [3H]arginine-labeled proinsulin described here definitely demonstrate the presence in the granules of an exopeptidase having properties similar to carboxypeptidase B. These experiments (Figs. 7 to 9) were designed to examine specifically the fate of the Arg-Arg (residues 64 and 65) sequence in the proinsulin preparations of a small but significant fraction of mono-arginyl insulin, i.e. insulin with a COOH-terminal arginine residue at position 81 of the B chain (39). Based on the model peptidase system described elsewhere (8) it is apparent that efficient conversion of proinsulin requires a relative excess of carboxypeptidase B-like activity in comparison with the trypsin activity and this may be a factor in our ability to detect this activity in the lysed granule preparations.

While these results all support the hypothesis that trypsin-like and carboxypeptidase B-like activities normally participate in the conversion of proinsulin to insulin, many important questions remain to be resolved. Among these are the nature of the proteolytic enzymes and their possible relationship to the known exocrine pancreatic enzyme counterparts, on the one hand, or to the lysosomal catheptic enzymes on the other. The possible membrane association of the endopeptidase activity mentioned...
earlier is an attractive, although still only speculative, possibility, as it might afford a means for protecting the insulin formed in the conversion reaction from further proteolytic degradation. Morphological studies of mature insulin secretion granules indicate that the dense central inclusion may consist mainly of crystalline insulin packed with repeat unit spacings that are closely similar to those observed in ordinary zinc insulin crystals (23). The mechanisms for accumulation of zinc in the secretion granules (41, 42), and its exact biochemical role are not known. Both proinsulin and insulin have been shown to bind zinc (43, 44), except in certain species such as the guinea pig and coypu (45), which lack the histidine residue at position 10 of the B chain required for zinc binding in the hexamer (46).

These experiments confirm that the C-peptide is retained in the granules after conversion (9, 47), presumably dissolved in the fluid space. However, the fate of the cationic arginine and lysine residues liberated during conversion is less clearly understood. Diffusion of these basic amino acids from the granule and their replacement by hydrogen ions may result in a slow decrease in pH within the granule which could favorably influence the crystallization of the insulin. Thus the pH in the external spaces of the rough endoplasmic reticulum, where proinsulin folding and thiol oxidation take place, may be slightly acidic. These ion movements may also favor the accumulation of zinc in the granules. Clearly, the biochemical processing of proinsulin and the formation of the mature secretory granule appear to be closely integrated processes which present many intriguing possibilities for further study.

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Studies on the Conversion of Proinsulin to Insulin: III. STUDIES IN VITRO WITH A CRUDE SECRETION GRANULE FRACTION ISOLATED FROM RAT ISLETS OF LANGERHANS
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